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(54) Title: BROAD SPECTRUM TUMOR SUPPRESSOR GENES, GENE PRODUCTS AND METHODS FOR TUMOR SUPPRESSION GENE THERAPY

(57) Abstract

The present invention relates to a broad-spectrum tumor suppressor gene and the protein expressed by that gene in appropriate hostcells. The protein is a second in-frame AUG codon-initiated retinoblasoma protein of about 94 kD relative molecular mass. The present invention also relates to methods of treating a mammal having a disease or disorder characterized by abnormal cellular proliferation, such as a tumor or cancer and methods of treating abnormally proliferating cells, such as tumor or cancer cells. Treatment is accomplished by inserting a host cell compatible $p94^{RB}$ expression vector or an effective amount of $p94^{RB}$ protein into a cell or cells in need of treatment.

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BROAD SPECTRUM TUMOR SUPPRESSOR GENES, GENE PRODUCTS AND METHODS FOR TUMOR SUPPRESSION GENE THERAPY

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1. BACKGROUND OF THE INVENTION

1.1 Field of the Invention

This invention is in the field of tumor suppressor genes (anti-oncogenes) and relates in general to products and methods for practicing broad-spectrum tumor suppressor gene therapy of various human cancers. In particular, the invention relates to methods for treating tumor cells (1) administering vectors comprising a nucleic acid sequence coding for a second in-frame AUG codon-initiated retinoblastoma protein of about 94 kD or (2) administering an effective amount of a protein coded for by the nucleic acid sequence.

1.2 Cancer

Cancers and tumors are the second most prevalent cause of death in the United States, causing 450,000 deaths per year. One in three Americans will develop cancer, and one in five will die of cancer (Scientific American Medicine, part 12, I, 1, section dated 1987). While substantial progress has been made in identifying some of the likely environmental and hereditary causes of cancer, the statistics for the cancer death rate indicates a need for substantial improvement in the therapy for cancer and related diseases and disorders.

1.3. Cancer Genes

A number of so-called cancer genes, i.e., genes that have been implicated in the etiology of cancer, have been identified in connection with hereditary forms of cancer and in a large number of well-studied tumor cells. Study of cancer genes has helped provide some understanding of the process of tumorigenesis. While a great deal more remains to be learned about cancer genes, the presently known cancer genes serve as useful models for understanding tumorigenesis.

Cancer genes are broadly classified into "oncogenes" which, when activated, promote tumorigenesis, and "tumor suppressor genes" which, when damaged, fail to suppress tumorigenesis. While these classifications provide a useful method for conceptualizing tumorigenesis, it is also possible that a particular gene may play differing roles depending upon the particular allelic form of that gene, its regulatory elements, the genetic background and the tissue environment in which it is operating.

1.3.1. Oncogenes

The oncogenes are somatic cell genes that are mutated from their wild-type alleles (the art refers to these wild-type alleles as protooncogenes) into forms which are able to induce tumorigenesis under certain conditions. There is presently a substantial literature on known and putative oncogenes and the various alleles of these oncogenes. In order to provide background information and to further the understanding of the scope of the invention, a brief discussion of representative oncogenes is provided.

For example, the oncogenes *ras* and *myc* are considered as models for understanding oncogenic processes in general. The *ras* oncogene is believed to encode a cytoplasmic protein, and the *myc* oncogene is believed to encode a nuclear protein. Neither the *ras*

oncogene nor the *myc* oncogene alone is able to induce full transformation of a normal cell into a tumor cell, but full tumorigenesis usually occurs when both the *ras* and *myc* oncogenes are present and expressed together in the same cell (Weinberg, R.A., 1989, Cancer Research 49:3713-3721, at page 3713). Such collaborative effects have been observed between a number of other studied oncogenes.

The collaborative model of oncogene tumorigenesis must be qualified by the observation that a cell expressing the *ras* oncogene that is surrounded by normal cells does not undergo full transformation. However, if most of the surrounding cells are also *ras*-expressing, then the *ras* oncogene alone is sufficient to induce tumorigenesis in a *ras*-expressing cell. This observation validates the multiple hit theory of tumorigenesis because a change in the tissue environment of the cell hosting the oncogene may be considered a second hit.

An alternative and equally valid hypothesis is that events that collaborate with the activation of an oncogene such as *ras* or *myc* may include the inactivation of a negative regulatory factor or factors (Weinberg, R.A., 1989, Cancer Research 49:3713-3721, at 3717; Goodrich, D.W. and Lee, W-H., 1992, Nature 360:177-179), i.e., a tumor suppressor protein.

1.3.2. Tumor Suppressor Genes

Tumor suppressor genes are genes that, in their wild-type alleles, express proteins that suppress abnormal cellular proliferation. When the gene coding for a tumor suppressor protein is mutated or deleted, the resulting mutant protein or the complete lack of tumor suppressor protein expression may fail to correctly regulate cellular proliferation, and abnormal cellular proliferation may take place, particularly if there is already existing damage to the cellular regulatory mechanism. A number of well-studied human tumors and tumor cell lines have been shown to have missing or nonfunctional tumor suppressor genes. Examples of tumor suppression genes include, but are not limited to, the retinoblastoma susceptibility gene or RB gene, the p53 gene, the deleted in colon carcinoma (DCC) gene and the neurofibromatosis type 1 (NF-1) tumor suppressor gene (Weinberg, R.A. Science, 1991, 254:1138-1146). Loss of function or inactivation of tumor suppressor genes may play a central role in the initiation and/or progression of a significant number of human cancers.

The list of putative tumor suppressor genes is large and growing. The following discussion of tumor suppressor genes is not intended to provide a complete review of all known and putative tumor suppressor genes, but is provided as background to indicate the state of the art and the problems to be overcome before the art is able to provide successful genetic therapy of diseases and disorders characterized by abnormally proliferating cells, e.g., tumor or cancer cells.

1.3.2.1. The Retinoblastoma Gene

The RB gene is one of the better studied tumor suppressor genes. The size of the RB gene complementary DNA (cDNA), about 4.7 Kb, permits ready

manipulation of the gene, so that insertions of the RB gene have been made into a number of cell lines. The RB gene has been shown to be missing or defective in a majority of retinoblastomas, sarcomas of the soft tissues and bones, and in approximately 20 to 40 percent of breast, lung, prostate and bladder carcinomas (Lee, W-H., et al., PCT Publ. No. WO 90/05180, at pages 38 and 39; *see also*, Bookstein, R. and Lee, W-H., 1991, Crit. Rev. Oncog., 2:211-217; Benedict, W.F. et al., J. Clin. Invest., 1990, 85:988-993).

Based upon study of the isolated RB cDNA clone, the predicted RB gene product has 928 amino acids and an expected molecular weight of 106 kD (Lee et al., 1987, Nature, 329:642-645). The natural factor corresponding to the predicted RB gene expression product has been identified as a nuclear phosphoprotein having an apparent relative molecular mass (Mr) of 110-114 kD (Lee et al., 1987, Nature, 329:642-645) or 110-116 kD (Xu et al., 1989, Oncogene 4:807-812). Hence, the literature generally refers to the protein encoded by the RB gene as $p110^{RB}$. In this connection, it is noteworthy that measurement of apparent relative molecular mass by SDS-PAGE is frequently inaccurate owing to protein secondary structure. Therefore, the full length RB protein of 928 amino acids is also referred to as the 115 kD (Yokota et al., 1988, Oncogene, 3:471-475), or 105 kD (*Whyte et al.*, 1988, Nature, 334:124-129) RB proteins. Various mutations of the RB gene are known. These are generally inactive. However, a 56 kD truncated RB protein, designated as $p56^{RB}$, that is considered to function in the same way as does $p110^{RB}$ retains activity (Goodrich et al., 1992, Nature 360:177-179).

On SDS-PAGE normal human cells show an RB protein pattern consisting of a lower sharp band with an Mr of 110 kD and a broader, more variable region above this

band with an Mr ranging from 110 kD to 116 kD. The 110 kD band is the underphosphorylated RB protein, whereas the broader region represents the phosphorylated RB protein. The heterogeneity of the molecular mass results from a varying degree of phosphorylation (Xu et al., 1989, Oncogene, 4:807-812).

The RB protein shows cyclical changes in phosphorylation. Most RB protein is unphosphorylated during G1 phase, but most (perhaps all) RB molecules are phosphorylated in S and G2 phases (Xu et al., 1989, Oncogene, 4:807-812; DeCaprio et al., 1989, Cell, 58:1085-1095; Buchkovich et al., 1989, Cell, 58:1097-1105; Chen et al., 1989, Cell, 58:1193-1198; Mihara et al., 1989, Science, 246:1300-1303). Furthermore, only the underphosphorylated RB protein binds to SV40 large T antigen. Given that RB protein binding by large T antigen is probably important for the growth promoting effects of large T antigen, this suggests that the underphosphorylated RB protein is the active form of the RB protein, and the phosphorylated RB protein in S and G2 phases is inactive (Ludlow et al., 1989, Cell, 56:57-65).

The RB gene expressing the first in-frame AUG codon-initiated RB protein is also referred to herein as the intact RB gene, the RB^{110} gene or the $p110^{RB}$ coding gene. It has also been observed that lower molecular weight (<100 kD, 98 kD, or 98-104 kD) bands of unknown origin which are immunoreactive to various anti-RB antibodies can be detected in immunoprecipitation and Western blots (Xu et al., 1989, Oncogene, 4:807-812; Furukawa et al., 1990, Proc. Natl. Acad. Sci., USA, 87:2770-2774; Stein et al., 1990, Science, 249:666-669).

Considering that the RB^{110} cDNA open reading frame sequence (McGee, T.L., et al., 1989, Gene, 80:119-128) reveals an in-frame second AUG codon located at exon

3, nucleotides 355-357, the deduced second AUG codon-initiated RB protein would be 98 kD, or 12 kD smaller than the $p110^{RB}$ protein. It has been proposed that the lower molecular weight bands are the underphosphorylated (98 kD) and phosphorylated (98-104 kD) RB protein translated from the second AUG codon of the RB mRNA (Xu et al., 1989, Oncogene, 4:807-812), although no data directly supported this hypothesis. Thus, no conclusive observation confirms the actual expression of the RB gene from the second in-frame AUG codon. Further, Sections 4.2.1, and Figure 5 *infra* provide data indicating the non-identity of the 98 kD protein bands of unknown origin and the second AUG codon-initiated protein products.

It has been proposed that introduction of a functional RB^{110} gene into an RB-minus tumor cell will likely "normalize" the cell. Of course, it is not expected that tumor cells which already have normal RB^{110} gene expression ("RB+") will respond to RB^{110} gene therapy, because it is presumed that adding additional RB expression cannot correct a non-RB genetic defect. In fact, it has been shown that in the case of RB+ tumor cell lines, such as the osteosarcoma cell line, U-2 OS, which expresses the normal $p110^{RB}$, introduction of an extra $p110^{RB}$ coding gene did not change the neoplastic phenotype of such tumor lines (Huang, et al., 1988, Science, 242:1563-1566).

In the only reported exception, introduction of a $p110^{RB}$ coding vector into normal human fibroblasts, WS1, which have no known RB or any other genetic defects, led to the cessation of cell growth (WO 91/15580, Research Development Foundation, by Fung et al., PCT application filed 10 April 1991, published 17 October 1991, at page 18). However, it is believed that these findings were misinterpreted since a plasmid, ppVUO-Neo, producing SV40 T antigen with a

well-known growth-promoting effect on host cells was used improperly to provide a comparison with the effect of RB^{110} expression on cell growth of transfected WS1 fibroblasts (Fung, et al. Id. *see* Example 2 page 25). This view is confirmed by the extensive literature, together with similar confirming data provided by the examples presented *infra*, clearly characterizing RB+ tumor cells as "incurable" by treatment with wild-type RB^{110} gene. In addition, it is noteworthy that the WS1 cell line per se is a generally recognized non-tumorigenic human diploid fibroblast cell line with limited cell division potential in culture. Therefore, WO91/15580 simply does not provide any method for effectively treating RB+ tumors with an RB^{110} gene. Thus, there remains a need for a broad-spectrum tumor suppressor gene for treating abnormally proliferating cells having any type of genetic defect.

1.3.2.2. The Neurofibromatosis Gene

Neurofibromatosis type 1 or von Recklinghausen neurofibromatosis results from the inheritance of a predisposing mutant allele or from alleles created through new germline mutations (C.J. Marshall, 1991, Cell, 64:313-326). The neurofibromatosis type 1 gene, referred to as the NF1 gene, is a relatively large locus exhibiting a mutation rate of around 10^{-4}. Defects in the NF1 gene result in a spectrum of clinical syndromes ranging from café-au-lait spots to neurofibromas of the skin and peripheral nerves to Schwannomas and neurofibrosarcomas.

The NF1 gene encodes a protein of about 2485 amino acids that shares structural similarity with three proteins that interact with the products of the *ras* protooncogene (Weinberg et al., 1991, Science, 254:1138-1146 at page 1141). For example, the NF1 amino acid sequence shows sequence homology to the

catalytic domain of *ras* GAP, a GTPase-activating protein for p21 *ras* (C.J. Marshall, 1991, Cell, 64:313-326 at pages 320 and 321).

The role of NF1 in cell cycle regulation is apparently a complex one that is not yet fully elucidated. For example, it has been hypothesized that it is a suppressor of oncogenically activated p21 *ras* in yeast (C.J. Marshall, (1991, Cell, 64:313-326, bridging pages 320 and 321, and citing to Ballester et al, 1990, Cell, 63:851-859). On the other hand, other possible pathways for NF1 interaction are suggested by the available data (C.J. Marshall, 1991, Cell, 64:313-326 at page 321; Weinberg et al., 1991, Science, 254:1138-1146 at page 1141).

At present, no attempts to treat NF1 cells with a wild-type NF1 gene have been undertaken due to the size and complexity of the NF1 locus. Therefore, it would be highly desirable to have a broad-spectrum tumor suppressor gene able to treat NF1 and any other type of cancer or tumor.

1.3.3.3. The p53 Gene

Somatic cell mutations of the p53 gene are said to be the most frequently mutated gene in human cancer (Weinberg et al., 1991, Science, 254:1138-1146 at page 1143). The normal or wild-type p53 gene is a negative regulator of cell growth, which, when damaged, favors cell transformation (Weinberg et al. *supra*). As noted for the RB protein, the p53 expression product is found in the nucleus, where it may act in parallel with or cooperatively with $p110^{RB}$. This is suggested by a number of observations, for example, both p53 and $p110^{RB}$ proteins are targeted for binding or destruction by the oncoproteins of SV40, adenovirus and human papillomavirus.

Tumor cell lines deleted for p53 have been successfully treated with wild-type p53 vector to

reduce tumorigenicity (Baker, S.J., et al., 1990, Science, 249:912-915). However, the introduction of either p53 or RB^{110} into cells that have not undergone lesions at these loci does not affect cell proliferation (Marshall, C.J., 1991, Cell, 64:313-326 at page 321; Baker, S.J., et al., 1990, Science, 249:912-915; Huang, H.-J.S., et al., 1988 Science, 242:1563-1566). Such experiments suggest that sensitivity of cells to the suppression of their growth by a tumor suppressor gene is dependent on the genetic alterations that have taken place in the cells. Such a dependency would be further complicated by the observation in certain cancers that alterations in the p53 tumor suppressor or gene locus appear after mutational activation of the *ras* oncogene (Marshall, C.J., 1991, Cell, 64:313-326; Fearon, E.R., and Vogelstein, B., 1990, Cell, 61:759-767).

Therefore, there remains a need for a broad-spectrum tumor suppressor gene that does not depend on the specific identification of each mutated gene causing abnormal cellular proliferation.

1.3.3.4. The Deleted in Colon Carcinoma Gene (DCC)

The multiple steps in the tumorigenesis of colon cancer are readily monitored during development by colonoscopy. The combination of colonoscopy with the biopsy of the involved tissue has uncovered a number of degenerative genetic pathways leading to the result of a malignant tumor. One well studied pathway begins with large polyps of which 60% of the cells carry a mutated, activated allele of K-*ras*. A majority of these tumors then proceed to the inactivation-mutation of the gene referred to as the deleted in colon carcinoma (DCC) gene, followed by the inactivation of the p53 tumor suppressor gene.

The DCC gene is a more than approximately one million base pair gene coding for a 190-kD transmembrane phosphoprotein which is hypothesized to be a receptor (Weinberg et al., 1991, Science, 254:1138-1146 at page 1141), the loss of which allows the affected cell a growth advantage. It has also been noted that the DCC has partial sequence homology to the neural cell adhesion molecule (Marshall, 1991, Cell, 64:313-326) which might suggest a role for the DCC protogene in regulating cell to cell interactions.

As can be appreciated, the large size and complexity of the DCC gene, together with the complexity of the K-*ras*, p53 and possibly other genes involved in colon cancer tumorigenesis demonstrates a need for a broad-spectrum tumor suppressor gene and methods of treating colon carcinoma cells which do not depend upon manipulation of the DCC gene or on the identification of other specific damaged genes in colon carcinoma cells.

1.4 Genetic Therapy: Gene Transfer Methods

The treatment of human disease by gene transfer has now moved from the theoretical to the practical realm. The first human gene therapy trial was begun in September 1990 and involved transfer of the adenosine deaminase (ADA) gene into lymphocytes of a patient having an otherwise lethal defect in this enzyme, which produces immune deficiency. The results of this initial trial have been very encouraging and have helped to stimulate further clinical trials (Culver, K.W., Anderson, W.F., Blaese, R.M., Hum. Gene. Ther., 1991, 2:107).

So far all but one of the approved gene transfer trials in humans rely on retroviral vectors for gene transduction. Retroviral vectors in this context are retroviruses from which all viral genes have been removed or altered so that no viral proteins are made

in cells infected with the vector. Viral replication functions are provided by the use of retrovirus 'packaging' cells that produce all of the viral proteins but that do not produce infectious virus. Introduction of the retroviral vector DNA into packaging cells results in production of virions that carry vector RNA and can infect target cells, but no further virus spread occurs after infection. To distinguish this process from a natural virus infection where the virus continues to replicate and spread, the term transduction rather than infection is often used.

The major advantages of retroviral vectors for gene therapy are the high efficiency of gene transfer into replicating cells, the precise integration of the transferred genes into cellular DNA, and the lack of further spread of the sequences after gene transduction (Miller, A.D., Nature, 1992, 357:455-460).

The potential for production of replication-competent (helper) virus during the production of retroviral vectors remains a concern, although for practical purposes this problem has been solved. So far, all FDA-approved retroviral vectors have been made by using PA317 amphotropic retrovirus packaging cells (Miller, A.D., and Buttimore, C., Molec. Cell Biol., 1986, 6:2895-2902). Use of vectors having little or no overlap with viral sequences in the PA317 cells eliminates helper virus production even by stringent assays that allow for amplification of such events (Lynch, C.M., and Miller, A.D., J. Viral., 1991, 65:3887-3890). Other packaging cell lines are available. For example, cell lines designed for separating different retroviral coding regions onto different plasmids should reduce the possibility of helper virus production by recombination. Vectors produced by such packaging cell lines may also provide

an efficient system for human gene therapy (Miller, A.D., 1992, Nature, 357:455-460).

Non-retroviral vectors have been considered for use in genetic therapy. One such alternative is the adenovirus (Rosenfeld, M.A., et al., 1992, Cell, 68:143-155; Jaffe, H.A. et al., 1992, Nature Genetics 1:372-378; Lemarchand, P. et al., 1992, Proc. Natl. Acad. Sci. USA, 89:6482-6486). Major advantages of adenovirus vectors are their potential to carry large segments of DNA (36 Kb genome), a very high titre (10^{11} ml^{-1}), ability to infect non-replicating cells, and suitability for infecting tissues in situ, especially in the lung. The most striking use of this vector so far is to deliver a human cystic fibrosis transmembrane conductance regulator (CFTR) gene by intratracheal instillation to airway epithelium in cotton rats (Rosenfeld, M.A., et al., Cell, 1992, 63:143-155). Similarly, herpes viruses may also prove valuable for human gene therapy (Wolfe, J.H., et al., 1992, Nature Genetics, 1:379-384). Of course, any other suitable viral vector may be used for genetic therapy with the present invention.

The other gene transfer method that has been approved by the FDA for use in humans is the transfer of plasmid DNA in liposomes directly to human cells in situ (Nabel, E.G., et al., 1990, Science, 249:1285-1288). Plasmid DNA should be easy to certify for use in human gene therapy because, unlike retroviral vectors, it can be purified to homogeneity. In addition to liposome-mediated DNA transfer, several other physical DNA transfer methods such as those targeting the DNA to receptors on cells by complexing the plasmid DNA to proteins have shown promise in human gene therapy (Wu, G.Y., et al., 1991, J. Biol. Chem., 266:14338-14342; Curiel, D.T., et al., 1991, Proc. Natl. Acad. Sci. USA, 88:8850-8854).

1.5 Proposed Strategies for Cancer Gene Therapy

It has been observed that certain tumor cells return to normal function when fused with normal cells, suggesting that replacement of a missing factor, such as a wild-type tumor suppressor gene expression product may serve to restore a tumor cell to a normal state (reviewed by Weinberg, R.A., 1989, Cancer Research 49:3713-3721, at 3717).

These observations have led to research aimed at providing genetic treatment of tumor cells having defective tumor suppressor genes. The proposed method of treatment requires identification of the damaged tumor suppressor gene, and introduction of the corresponding undamaged gene (including a promoter and a complete encoding sequence) into the affected tumor cells by means of a vector such as a retrovirus able to express the gene product. It is proposed that the incorporated functional gene will convert the target cell to a non-malignant state.

For example, The Regents of the University of California, in Patent Cooperation Treaty patent application (by Lee et al., number WO 90/05180, having an international filing date of 30 October 1989 and published 17 May 1990), disclose a scheme for identifying an inactive or defective tumor suppressor gene and then replacing such a defective gene with its functional equivalent. In particular, the WO 90/05180 application proposes, based on in vitro studies, to insert a functional RB^{110} gene into an RB-minus tumor cell by means of a retroviral vector in order to render such cells non-malignant.

In addition, international application WO 89/06703 (by Dryja et al., having an international filing date of 23 January 1989, and published 27 July 1989) proposes the treatment of retinoblastoma defective tumors by administering a retinoblastoma gene expression product.

In this connection, it has been reported that the introduction of the RB^{110} gene into RB-minus retinoblastoma, osteosarcoma, bladder and prostate carcinoma cells resulted in cells showing reduced tumorigenicity in nude mice, but probably not a reduced cell growth rate. The results varied depending on the particular parental cell line (Goodrich et al., 1992, Cancer Research 52:1968-1973; Banerjee, A., et al., 1992, Cancer Research, 52:6297-6304; Takahashi, R., et al., 1991, Proc. Natl. Acad. Sci., USA, 88:5257-5261; Xu, H-J., et al., 1991, Cancer Research, 51:4481-4485; Bookstein et al, 1990, Science, 247:712-715; Huang, H-J.S., et al., 1988, Science 242, 1563-1566). However, the suppression of tumorigenicity by introduction of the $p110^{RB}$ coding gene into RB-minus tumor cells is incomplete. The $p110^{RB}$ reconstituted tumor cells still form invasive tumors in nude mice (Xu, H-J., et al., 1991, Cancer Research, 51:4481-4485; Takahashi, R., et al., 1991, Proc. Natl. Acad. Sci., USA, 88:5257-5261; Banerjee, A., et al., 1992, Cancer Research, 52:6297-6304). In particular, it has been shown that $p110^{RB}$ reconstituted retinoblastoma cells inoculated into an orthotopic site (in this instance, the eye) consistently produced tumors (Xu, H-J., et al., 1991, Cancer Research 51:4481-4485). These findings, which will be discussed in detail *infra*, caution that the tumor suppressor gene replacement therapy as heretofore envisioned may simply result in cells that only appear to be "cured". Certainly, the findings of Xu et al. indicate a need for an improved genetic therapy for tumors which avoids these shortcomings.

Another proposed method of treating cancer by gene therapy is to antagonize the function of an oncogene by placing an artificial gene, constructed to have an inverted nucleotide sequence compared to the oncogene, into a tumor cell (U.S. patent number

4,740,463, issued April 26, 1988 by Weinberg, et al.).

All of these proposed solutions also share the deficiency of requiring that the specific genetic defect of the tumor to be treated be identified prior to treatment.

Since the $p110^{RB}$ protein product is active in the underphosphorylated state (discussed in detail *supra*), and phosphoamino acid analysis has demonstrated only phosphoserine and phosphothreonine but not phosphotyrosine in RB protein (Shew, J-Y., et al., 1989, Oncogene Research, 1:205-213), it has been proposed to make a mutant RB protein with its serine or threonine residues being replaced by alanine or valine or others and that introduction of such a mutant, unphosphorylated RB protein into target cells may lead to growth arrest (International Application WO 91/15580, Research Development Foundation, by Fung et al., at page 20). Unfortunately, in all cases analyzed so far, the human RB protein carrying a point mutation and retaining the unphosphorylated state were invariably inactive proteins and associated with tumorigenesis rather than tumor suppression (Templeton et al., 1991, Proc. Natl. Acad. Sci., USA, 88:3033-3037.

1.6 Tumor Suppressor Gene Resistance

As the above discussion of gene mutations in tumor cells has indicated, not every cancer gene is a suitable candidate for wild-type gene replacement therapy due to the gene size or complexity or for other reasons. The retinoblastoma gene is one of those tumor suppressor genes that is readily accessible to study, thus it provides a model for understanding some of the other disadvantages to cancer gene replacement therapy as heretofore understood.

It is known that reintroduction of the retinoblastoma tumor suppressor gene into RB-defective tumor cells inhibits the tumor cell growth and suppresses the neoplastic phenotype of the target cells (WO 90/05180, cited *supra*; Huang et al., 1988, Science, 242:1563-1566; Bookstein et al., 1990, Science, 247:712-715; Xu et al., 1991, Cancer Res., 51:4481-4485; Takahashi et al., 1991, Proc. Natl. Acad. Sci., USA, 88:5257-5261; Goodrich et al., 1992, Cancer Res., 52:1968-1973; Banerjee et al., 1992, Cancer Res., 52:6297-6304).

However, the suppression of tumorigenicity is often incomplete. A significant percentage of the RB-reconstituted tumor cells still form small tumors after a longer latency period in nude mouse tumorigenicity assays. Such tumors, although retaining normal RB expression, are histologically malignant and invasive (Xu et al., 1991, Cancer Res., 51:4481-4485; Takahashi et al., 1991, Proc. Natl. Acad. Sci., USA, 88:5257-5261; Banerjee et al., 1992, Cancer Res., 52:6297-6304).

Furthermore, it has been observed that several cell lines derived from such RB-positive tumors have become very tumorigenic and have formed large, progressively growing tumors when subsequently injected into nude mice (Zhou, Y.; Li, J.; Xu, K.; Hu, S-X.; Benedict, W.F., and Xu, H-J., Proc. Am. Assoc. Cancer Res., 34:3214, 1993). This phenomenon, which is referred to herein as tumor suppressor gene resistance (TSGR) is a serious obstacle to the successful implementation of any scheme of tumor suppressor gene therapy for human cancers.

Without wishing to be bound by any particular hypothesis or explanation of the TSGR phenomenon, it is believed that the RB gene product exemplifies a possible explanation for TSGR. RB proteins have an active form (underphosphorylated protein) and an

inactive form (phosphorylated protein). Therefore, RB-positive tumor cells may have inherited or acquired the ability to phosphorylate RB proteins to the inactive state and allow tumor cell proliferation to continue. Thus, conversion of RB-minus cells with plasmid or virus vectors coding for the $p110^{RB}$ protein provides only incomplete suppression, or even exacerbation of a percentage of the malignant cell population because the $p110^{RB}$ protein remains phosphorylated and inactive in some of the target cells.

Alternatively, the tumor cells expressing the RB^{110} gene may simply have again inactivated the RB^{110} gene by mutation in subsequent cell divisions (Lee et al., 1990, Immunol. Ser. 51:169-200, at page 188). Thus, there remains a need for a method of treating tumor cells by gene therapy so that the possibility of further mutation and resurgence of malignancy is avoided.

1.7 Summary of Obstacles to Cancer Gene Therapy

In brief, there are at least three major obstacles to be overcome to achieve a practical tumor suppressor gene therapy for tumor cells:

1) The necessity to determine the identity and sequence of each defective tumor suppressor gene or oncogene before attempting genetic therapy of that tumor. This is particularly a problem considering the multiple genetic defects found in many tumor cells studied;

2) The size and complexity of certain tumor suppressor genes or oncogenes renders manipulation of certain of these genes difficult; and

3) The possibility that TSGR as described above for the RB^{110} model system will generate tumor cells

that have equal or greater dysfunction than did the original abnormal cells.

Accordingly, there is a need in the art for a genetic therapy for tumor or cancer cells which can safely overcome these problems and provide an effective treatment for all types of tumor cells without the need to determine the exact genetic deficiency of each treated tumor cell and without the risk of TSGR resurgence and exacerbation of the malignancy.

2. SUMMARY OF THE INVENTION

Obstacles to the successful practice of tumor suppressor gene therapy of cancers are avoided by the present invention. In a totally unexpected and surprising discovery, it has been determined that the second in-frame AUG codon-initiated retinoblastoma suppressor protein of about 94 kD ($p94^{RB}$) is a broad-spectrum tumor suppressor, and that insertion of a gene capable of expressing this protein, or the protein itself, into an abnormally proliferating cell, such as a cancer or tumor cell, causes that cell to enter a senescent-like state, terminating the proliferation. The cell so-treated simply stops replicating and dies. The cell may possess any type of genetic defect, known or unknown, so that there is no need to determine the exact nature of the genetic defect associated with the abnormal proliferation. Further, the population of treated cells exhibits an unexpectedly much lower incidence of TSGR resurgence and exacerbation of malignancy than do cells treated with any other tumor suppressor gene. The method is repeated as needed.

Therefore, the invention provides $p94^{RB}$ encoding vectors and $p94^{RB}$ proteins for use in treatment of tumors or cancers, and methods of preparing $p94^{RB}$ proteins suitable for use in methods of treatment.

The invention also provides methods of treatment for mammals such as humans, as well as methods of treating abnormally proliferating cells, such as cancer or tumor cells. Broadly, the invention contemplates treating abnormally proliferating cells, or mammals having a disease characterized by abnormally proliferating cells by any suitable method known to permit a host cell compatible $p94^{RB}$ encoding vector or a $p94^{RB}$ protein to enter the cells to be treated so that suppression of proliferation is achieved.

In one embodiment, the invention comprises a method of treating a disease characterized by abnormally proliferating cells, in a mammal, by administering an expression vector coding for $p94^{RB}$ to the mammal having a disease characterized by abnormally proliferating cells, inserting the expression vector into the abnormally proliferating cells, and expressing $p94^{RB}$ in the abnormally proliferating cells in an amount effective to suppress proliferation of those cells. The expression vector is inserted into the abnormally proliferating cells by viral infection or transduction, liposome-mediated transfection, polybrene-mediated transfection, CaPO4 mediated transfection and electroporation. The treatment is repeated as needed.

In another embodiment, the invention comprises a method of treating abnormally proliferating cells of a mammal by inserting a $p94^{RB}$ encoding expression vector into the abnormally proliferating cells and expressing $p94^{RB}$ therein in amounts effective to suppress proliferation of those cells. The treatment is repeated as needed.

In another alternative embodiment, the invention provides a DNA molecule able to suppress growth of an abnormally proliferating cell. The DNA molecule encodes a $p94^{RB}$ protein having an amino acid sequence

substantially according to SEQ ID NO:3, provided that the DNA molecule does not also code for a $p110^{RB}$ protein. In a more preferred embodiment, the DNA molecule has the DNA sequence of SEQ ID NO:1, and is expressed by an expression vector. The expression vector may be any host cell-compatible vector. The vector is preferably selected from the group consisting of a retroviral vector, an adenoviral vector and a herpesviral vector.

In another alternative embodiment, the invention provides a $p94^{RB}$ protein having an amino acid sequence substantially according to SEQ ID NO:3.

In another alternative embodiment, the invention provides a method of producing a $p94^{RB}$ protein by the steps of: inserting a compatible expression vector comprising a $p94^{RB}$ encoding gene into a host cell and causing the host cell to express $p94^{RB}$ protein.

In another alternative embodiment, the invention comprises a method of treating abnormally proliferating cells of a mammal ex vivo by the steps of: removing a tissue sample in need of treatment from a mammal, the tissue sample comprising abnormally proliferating cells; contacting the tissue sample in need of treatment with an effective dose of an $p94^{RB}$ encoding expression vector; expressing the $p94^{RB}$ in the abnormally proliferating cells in amounts effective to suppress proliferation of the abnormally proliferating cells. The treatment is repeated as necessary; and the treated tissue sample is returned to the original or another mammal. Preferably, the tissue treated ex vivo is blood or bone marrow tissue.

In another alternative embodiment, the invention comprises a method of treating a disease characterized by abnormal cellular proliferation in a mammal by a process comprising the steps of administering $p94^{RB}$ protein to a mammal having a disease characterized by

abnormally proliferating cells, such that the $p94^{RB}$ protein is inserted into the abnormally proliferating cells in amounts effective to suppress abnormal proliferation of the cells. In a preferred embodiment, the $p94^{RB}$ protein is liposome encapsulated for insertion into cells to be treated. The treatment is repeated as necessary.

In another alternative embodiment the invention comprises a method of treating abnormally proliferating cells of a mammal ex vivo by a process comprising the steps of removing a tissue sample in need of treatment from a mammal, the tissue sample comprising abnormally proliferating cells contacting the tissue sample in need of treatment with an effective dose of a $p94^{RB}$ protein. The treatment is repeated as necessary, and then the treated tissue is returned to the mammal or placed into another mammal.

In a more preferred embodiment the tumor or cancer cells to be treated are cells having one or more genetically defective tumor suppressor genes and oncogenes selected from the group consisting of an RB, a p53, a c-myc, an N-ras and a c-yes-1 gene.

In a more preferred embodiment the tumor or cancer cells are cells having no detectable genetic defect of a tumor suppressor gene selected from the group consisting of an RB gene and a p53 gene.

In a still more preferred embodiment the tumor or cancer cells are lung carcinoma cells.

In a still more preferred embodiment the $p94^{RB}$ encoding expression vector or the $p94^{RB}$ protein are administered by means of aerosol delivery of liposome-encapsulated $p94^{RB}$ encoding expression vector or $p94^{RB}$ protein into a lung in need of such treatment.

3. DETAILED DESCRIPTION OF THE INVENTION

3.1 Definitions

The terms "cancer" or "tumor" are clinically descriptive terms which encompass a myriad of diseases characterized by cells that exhibit unchecked and abnormal cellular proliferation. The term "tumor", when applied to tissue, generally refers to any abnormal tissue growth, i.e., excessive and abnormal cellular proliferation. A tumor may be "benign" and unable to spread from its original focus, or "malignant" and capable of spreading beyond its anatomical site to other areas throughout the hostbody. The term "cancer" is an older term which is generally used to describe a malignant tumor or the disease state arising therefrom. Alternatively, the art refers to an abnormal growth as a neoplasm, and to a malignant abnormal growth as a malignant neoplasm.

Irrespective of whether the growth is classified as malignant or benign, the causes of excessive or abnormal cellular proliferation of tumor or cancer cells are not completely clear. Nevertheless, there is persuasive evidence that abnormal cellular proliferation is the result of a failure of one or more of the mechanisms controlling cell growth and division. It is also now believed that the mechanisms controlling cell growth and division include the genetic and tissue-mediated regulation of cell growth, mitosis and differentiation. These mechanisms are thought to act at the cell nucleus, the cell cytoplasm, the cell membrane and the tissue-specific environment of each cell. The process of transformation of a cell from a normal state to a condition of excessive or abnormal cellular proliferation is called tumorigenesis.

It has been observed that tumorigenesis is usually a multistep progression from a normal cellular state to, in some instances, a full malignancy. It is

therefore believed that multiple "hits" upon the cell regulatory mechanisms are required for full malignancy to develop. Thus, in most instances, it is believed that there is no single cause of excessive proliferation, but that these disorders are the end result of a series of cumulative events.

While a malignant tumor or cancer capable of unchecked and rapid spread throughout the body is the most feared and usually the deadliest type of tumor, even so-called benign tumors or growths can cause significant morbidity and mortality by their inappropriate growth. A benign tumor can cause significant damage and disfigurement by inappropriate growth in cosmetically sensitive areas, or by exerting pressure on central or peripheral nervous tissue, blood vessels and other critical anatomical structures.

A broad-spectrum tumor suppressor gene is a genetic sequence coding for a protein that, when inserted into and expressed in an abnormally proliferating host cell, e.g., a tumor cell, suppresses abnormal proliferation of that cell irrespective of the cause of the abnormal proliferation. The second in-frame AUG (ATG in DNA) codon-initiated retinoblastoma gene disclosed herein exemplifies such a broad-spectrum tumor suppressor gene and is referred to herein as the $p94^{RB}$ coding gene, as the RB^{94} gene or as a DNA molecule coding for pRB^{94}. According to the nucleotide sequence of the retinoblastoma susceptibility gene (McGee, T.L., et al., 1989, Gene, 80:119-128), the $p94^{RB}$ coding gene comprises the nucleotide sequence from exon 3, nucleotide 355 to exon 27, nucleotide 264. Thus, the $p94^{RB}$ encoding gene by definition excludes that portion of the RB^{110} gene upstream from the second in-frame AUG start codon. Figures 1A-1F show the DNA sequence of

the RB^{94} gene wherein the ATG codon begins at nucleotide 19 of that figure (SEQ ID NO:1; SEQ ID NO:2).

A broad-spectrum tumor suppressor protein (including phosphoproteins, lipoproteins, glycoproteins and other protein-based derivatives) is a substance that when injected into, absorbed by or caused to be expressed in any abnormally proliferating cell, reduces or completely suppresses abnormal cellular proliferation. The protein expressed by the second in-frame AUG codon-initiated retinoblastoma gene disclosed herein exemplifies such a broad-spectrum tumor suppressor protein. It is a phosphoprotein of about 94 kD relative molecular mass, and is also referred to herein as $p94^{RB}$ (SEQ ID NO:3).

One of ordinary skill in the art will be able to determine if any other fragment of a tumor suppressor protein, e.g., the third or fourth AUG codon-initiated retinoblastoma protein of about 90 kD and 83 kD, respectively, also has the property of suppressing abnormal cellular proliferation.

3.2 Brief Description of the Figures

Figures 1A-1F: Nucleotide sequence of the cDNA fragment encoding the 94 kD therapeutic RB protein (plus strand is SEQ ID NO:1, minus strand is SEQ ID NO:2).

Figures 2A-2F: Amino acid sequence of the 94 kDa therapeutic RB protein (SEQ ID NO:3).

Figure 3: Construction of baculovirus expression vector for the 94 kDa therapeutic RB protein synthesis; *R.S. is recombination sequence.

Figures 4A and 4B:Intracellular localization of recombinant baculovirus-produced $p110^{RB}$ and $p94^{RB}$ in insect cells: Figure 4A shows mock-infected Sf9 cells; Figure 4B shows cells producing $p110^{RB}$; and Figure 4C shows cells producing $p94^{RB}$; note that

protein is localized to the nucleus in Figures 4B and 4C. Protein localization is by anti-RB immunochemical staining.

Figure 5: A diagram of complex formation of baculovirus-expressed and subsequently purified $p110^{RB}$ and $p94^{RB}$ proteins with SV40 T antigen. The immunoaffinity chromatography purified proteins were mixed with an equal amount of T antigen, and aliquots of the mixture were immunoprecipitated with PAB419 anti-T antibody, followed by Western blotting. The blot was sequentially incubated with MAb-1 anti-RB antibody and PAB419 antibody. Lane 1, lysate of T antigen immortalized W138 VA13 fibrobrasts was used as a control; lane 2, purified $p110^{RB}$; lane 3, co-precipitation of T-Ag with $p110^{RB}$; lane 4, purified $p94^{RB}$; lane 5, co-precipitation of T-Ag with $p94^{RB}$.

Figures 6A and 6B: Construction of recombinant plasmids for high-level expression of $p110^{RB}$ (pCMV-f-RB35) and $p94^{RB}$ (pCMV-s-RB42) proteins in human cells using cytolomegalovirus promoter/enhancer: Figure 6A is an explanatory drawing of the $p110^{RB}$ coding cDNA; Figure 6B provides maps of the $p110^{RB}$ and $p94^{RB}$ expression plasmids where pCMV-f-RB35 codes for $p110^{RB}$ and pCMV-s-RB42 codes for $p94^{RB}$. Note that pCMV-s-RB42 has most of $p110^{RB}$ coding region deleted upstream of the second ATG.

Figures 7A and 7B: Construction of recombinant plasmids for expression of $p110^{RB}$ (pβA-f-RB33) and $p94^{RB}$ (pβA-s-RB34) proteins in human cells using ß-actin promoter: Figure A is a map of the $p110^{RB}$ coding plasmid, pβA-f-RB33; Figure B is a map of the $p94^{RB}$ coding plasmid, pβA-s-RB34. Note that pβA-s-RB34 has most of the $p110^{RB}$ coding region deleted upstream of the second ATG.

Figures 8A, 8B and 8C: Morphological effects of $p110^{RB}$ and $p94^{RB}$ expression on RB-defective bladder carcinoma cell line 5637 (ATCC HTB9): Figure 8A is

mock-transfected HTB9 cells; Figure 8B is $p110^{RB}$ expressing HTB9 transfectants; Figure 8C is $p94^{RB}$-expressing HTB9 transfectants. Arrows indicate examples for RB-positive immunostained cells. Note that the $p110^{RB}$ expressing cells of Figure 8B appear normal, but that the $p94^{RB}$ expressing cells of Figure 8C are senescent.

Figure 9. Half-life analysis of $p110^{RB}$ and $p94^{RB}$ proteins in RB-reconstituted bladder carcinoma cell line, 5637. The bladder tumor cells were transfected in multiple dishes with either $p110^{RB}$ (pβA-f-RB33) or $p94^{RB}$ (pβA-s-RB34) expression plasmids. Twenty-four hours after transfection the cells were labeled with [^{35}S]-methionine and chased with excess unlabeled methionine for 0, 6, 12 and 24 hours, respectively. The $p110^{RB}$ and $p94^{RB}$ proteins were determined by immunoprecipitation: the left side of the figure (0-12 hours) shows the half-life of $p110^{RB}$ is less than 6 hours; the right side of the figure (0-24 hours) shows the half-life of $p94^{RB}$ is about 12 hours.

Figure 10. Western blot analysis of exogenous $p110^{RB}$ and $p94^{RB}$ proteins in transiently transfected 5637 cells showing the distinct underphosphorylation state of the $p94^{RB}$ protein: lane 1 shows normal human fibroblast cell line, WI-38; lane 2 shows parental RB-minus bladder carcinoma cell line, 5637; lane 3 shows 5637 cells transfected with $p110^{RB}$-expressing plasmid; lane 4 shows 5637 cells transfected with $p94^{RB}$-expressing plasmid.

Figures 11A-11C. Expression of the human full-length RB protein, $p110^{RB}$ (Figure 11B) and the broad-spectrum tumor suppressor protein, $p94^{RB}$ (Figure 11C) in normal (non-tumorigenic) mouse fibroblast cells via retrovirus plasmid vectors. Both the $p110^{RB}$- and $p94^{RB}$-expressing cells (arrows) have normal viable morphology similar to the parental cells Figure 11A).

Figures 12A-12D. Expression of the human $p110^{RB}$ (Figure 12C) and $p94^{RB}$ (Figure 12D) proteins in mouse urinary bladder mucous membranes *in vivo*. Liposomes were mixed with the $p110^{RB}$- and $p94^{RB}$- expressing plasmids, respectively and infused directly into the mouse bladder via a catheter. Notably, transitional epithelia expressing the $p110^{RB}$ (Figure 12C, arrows) or $p94^{RB}$ (Figure 12D, arrows) both retained normal, viable morphology similar to their counterparts in the untreated mouse bladder (Figure 12A) or the mouse bladder treated with liposomes only (Figure 12B).

3.3 The Invention

The present invention is based upon the unexpected discovery that $p94^{RB}$ expressed by an expression vector in any abnormally proliferating target cell, e.g., a cancer or tumor cell, causes the suppression of the abnormal proliferation. Surprisingly, the treatment has been effective with all tested tumor cell lines and is not limited to treatment of RB-minus tumor cells.

Without wishing to be bound by a particular hypothesis or proposed mechanism of action, it is believed that the $p94^{RB}$ protein remains in the active, underphosphorylated form, and has a half-life in the target cell which is two to three times longer than that of $p110^{RB}$. Thus, it is possible that a synergistic combination of accumulation of $p94^{RB}$ together with its tendency to remain in an underphosphorylated, active form serves to terminate the cell replication cycle in target tumor cells. However, whatever the mechanism of action, the property of suppressing cell growth and inducing senescence or killing any abnormally proliferating cell, irrespective of its genetic defect, is nevertheless completely unanticipated and unexpected.

In order to obtain the broad-spectrum tumor suppressor protein, a gene coding for the second in-frame AUG codon-initiated RB protein, i.e., $p94^{RB}$, was expressed by a baculovirus vector in insect host cells as a stable nuclear phosphoprotein. The resulting unphosphorylated forms of $p94^{RB}$ were able to form a specific complex with SV40 T antigen, providing an important verification that the $p94^{RB}$ protein shares many functional properties of the naturally occurred $p110^{RB}$ protein, i.e., phosphorylation, viral oncoprotein association and nuclear tethering (Templeton et al., 1991, Proc. Natl. Acad. Sci., USA, 88:3033-3037).

The effects of transfection by either first or second in-frame AUG codon-initiated RB protein expression plasmid were compared on a number of well known human tumor cell lines. The tested cell lines included: an RB-defective human bladder carcinoma cell line, 5637 (ATCC HTB9); RB-defective human breast carcinoma cell line, MDA-MB-468 (ATCC HTB132); RB-defective human non-small cell lung carcinoma cell line, H2009 (Kratzke, R.A., et al., 1992, The Journal of Biological Chemistry, 267:25998-26003); RB-defective human prostate carcinoma cell line, DU145 (ATCC HTB81); RB-defective human osteosarcoma cell line, Saos-2 (ATCC HTB85); RB-defective human fibrosarcoma metastatic to lung cell line, Hs913T (ATCC HTB152); human cervix adenocarcinoma cell line, HeLa (ATCC CCL2) and human fibrosarcoma cell line, HT1080 (ATCC CCL121). Both the HeLa and HT1080 cell lines have normal $p110^{RB}$ expression. Each of these cell lines were separately transfected with the $p110^{RB}$ coding and the $p94^{RB}$ coding expression plasmids. The results demonstrated that the second in-frame AUG codon-initiated RB protein, $p94^{RB}$, was a more effective cell growth inhibitor, causing those dividing tumor

cells to senesce and die. On the other hand, most normal human cells in vivo are either non-dividing or have the potential to progress into the cell cycle after a long latency period. Therefore, $p94^{RB}$, as an active cell cycle regulatory factor and a therapeutic reagent is expected to show little or no toxicity when transiently expressed in normal cells in vivo.

The study also demonstrated that the RB-minus tumor cells expressing the second in-frame AUG codon-initiated RB protein, $p94^{RB}$, did not progress through the cell cycle, as evidenced by their failure to incorporate [^{3}H]-thymidine into DNA. However, the percentage of cells undergoing DNA replication was only slightly lower in cells producing the intact RB protein ($p110^{RB}$) than in cells that were RB-negative.

Of particular interest was the fact that the RB-defective bladder carcinoma cell line, 5637, failed to phosphorylate the second in-frame AUG codon-initiated RB protein as shown by Western blot analysis. In contrast, the intact RB protein ($p110^{RB}$) expressed in transfected 5637 cells were fully phosphorylated. Moreover, the half-life of the second in-frame AUG codon-initiated RB protein, $p94^{RB}$, was shown to be two- to three-fold greater than the intact RB protein ($p110^{RB}$). Therefore, the accumulation of only unphosphorylated (active) $p94^{RB}$ proteins may account for the failure of transiently transfected 5637 tumor cells to enter S phase, and this in turn may cause these tumor cells to senesce and die.

In addition, it has also been found that $p94^{RB}$ protein has its preferentially associated cellular proteins as compared to 100^{RB}. This difference in associated proteins may also contribute to the unique broad-spectrum tumor cell growth suppressing functions of $p94^{RB}$ protein.

Both the fibrosarcoma cell line, HT1080 and cervix carcinoma cell line, HeLa, which have normal RB gene expression, were also successfully treated with the second in-frame AUG codon-initiated RB protein ($p94^{RB}$) expression plasmid, demonstrating that expression of the $p94^{RB}$ protein in RB+ cancer or tumor cells significantly suppressed the tumor cell growth. Therefore, an advantage of the present invention is that the methods and products herein disclosed can be used for therapeutic treating tumors having no specific tumor suppressor gene defects, which provides a significant advantage over previous techniques for human tumor suppressor gene therapy.

Table 1, on the following page, provides a summary of the identification of the tested tumor cell lines, their tumor origin and genetic defects.

TABLE 1: The Status of Antioncogenes (Tumor Suppressor Genes) and Oncogenes in $p94^{RB}$-Treated Human Tumor Cells

RECIPIENT CELLS	TUMOR ORIGIN	ANTIONCOGENES		ONCOGENES
		RB	p53	
5637	Bladder carcinoma, primary tumor	Negative	Mutation	
DU145	Prostate carcinoma, metastasis to brain	Point mutation	Mutation	
MDA-MB-468	Breast Carcinoma	Large deletion	Mutation	
H2009	Lung carcinoma	Mutation	Mutation	
Hs913T	Fibrosarcoma, metastasis to lung	Large deletion	Negative	
Saos2	Osteosarcoma, primary tumor	Large deletion	Negative	
HeLa	Cervix carcinoma, primary tumor	Normal	Negative	*c-myc* activation[1]
HT1080	Fibrosarcoma, primary tumor	Normal	Normal	N-*ras* and *c-yes*-1 activation[2],[3]

1 Durst, M., et al. Papillomavirus sequences integrate near cellular oncogenes in some cervical carcinomas. Proc. Natl. Acad. Sci., USA, 84(4):1070-1074, 1987.

2 Brown, R., et al. A mechanism of activation of an N-*ras* gene in the human fibrosarcoma cell line HT1080. EMBO J., 3:1321-1326, 1984.

3 Sugawara, K., et al. Distribution of *c-y s*-1 gene product in various cells and tissues. Br. J. Cancer, 63(4):508-513, 1991.

3.3.1. Preparation of RB^{94} Vectors

3.3.1.1. Therapeutic Vectors

Any of the methods known to the art for the insertion of DNA fragments into a vector, as described, for example, in Maniatis, T., Fritsch, E.F., and Sambrook, J. (1989): Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; and Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1992): Current Protocols in Molecular Biology, John Wiley & Sons, New York, may be used to construct $p94^{RB}$ encoding gene expression vectors consisting of appropriate transcriptional/translational control signals and the desired RB cDNA sequence downstream from the first in-frame AUG codon, that is unable to code for $p110^{RB}$. These methods may include *in vitro* DNA recombinant and synthetic techniques and *in vivo* genetic recombination. Expression of a nucleic acid sequence encoding a $p94^{RB}$ may be regulated by a second nucleic acid sequence so that the $p94^{RB}$ is expressed in a host infected or transfected with the recombinant DNA molecule. For example, expression of $p94^{RB}$ may be controlled by any promoter/enhancer element known in the art. The promoter activation may be tissue specific or inducible by a metabolic product or administered substance.

Promoters/enhancers which may be used to control $p94^{RB}$ gene expression include, but are not limited to, the native RB promoter, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama, H., et al., 1989, J. Exp. Med., 169:13), the human ß-actin promoter (Gunning, P., et al., 1987, Proc. Natl. Acad. Sci. USA, 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig, D.F., et al.,

1984, Mol. Cell Biol., 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss, R., et al., 1985, RNA Tumor Viruses, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early region promoter (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, Cell 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), the adenovirus promoter (Yamada et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82(11):3567-71), and the herpes simplex virus LAT promoter (Wolfe, J.H., et al., 1992, Nature Genetics, 1:379-384).

Expression vectors compatible with mammalian host cells for use in genetic therapy of tumor or cancer cells, include, but are not limited to: plasmids, retroviral vectors, adenovirus vectors, herpes viral vectors, and non-replicative avipox viruses, as disclosed, for example, by U.S. Patent No. 5,174,993.

In a specific embodiment, a plasmid vector derived from pHßAPr-1-neo, was constructed for expression of $p94^{RB}$ in mammalian cells by placing the coding sequence for $p94^{RB}$ under control of the human ß-actin gene promoter (Gunning, P. et al., Proc. Natl. Acad. Sci., USA, 1987, 84:4831-4835).

In another specific embodiment, a plasmid vector derived from pCMV-Neo-Bam (Baker, S.J., et al., Science, 1990, 249:912-915), was constructed for expression of $p94^{RB}$ in mammalian cells by placing the coding sequence for $p94^{RB}$ under control of the cytomegalovirus (CMV) promoter/enhancer sequences.

In another specific embodiment, a retroviral vector, pLLRNL (Miller, A.D., et al., 1985, Proc.

Natl. Acad. Sci., USA, 5:431) is used to construct a vector able to transduce mammalian cells and express $p94^{RB}$ protein under the control of the MuLV LTR promoter, the CMV promoter, the ß-actin promoter or any other effective promoter.

In yet another specific embodiment, an adenovirus type 5 (Ad5) deletion mutant, Ad-d1324, and a plasmid, pTG5955 (Rosenfeld, M.A., et al., Cell, 1992, 68:143-155) are used to construct an adenovirus vector able to infect mammalian cells and express $p94^{RB}$ protein under the control of the adenovirus type 2 (Ad2) major late promoter, the CMV promoter, the ß-actin promoter or any other effective promoter.

3.3.1.2. Vectors for Production and Purification of $p94^{RB}$ Protein

Alternatively, expression vectors compatible with host cells suitable for production of $p94^{RB}$ may be constructed to express $p94^{RB}$ protein in those compatible host cells. These include but are not limited to mammalian cells infected with a virus (e.g., adenovirus, retrovirus, herpes simplex virus, avipox virus); insect cells infected with a virus (e.g., baculovirus); microorganisms such as yeasts containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA. The expression controlling elements of vectors vary in their strengths and specifications. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. The produced $p94^{RB}$ may be purified from host cells by affinity chromatography, electrophoresis, high-performance liquid chromatography (HPLC) or any other methods known to the art.

In a specific embodiment an engineered derivative of Autographa california Multiple Nuclear Polyhedrosis

Virus ("AcMNPV") was used to produce $p94^{RB}$ protein in cultured Fall Army worm Spondoptera frugiperda cells (Sf9 cells) with a strong temporally regulated promoter of the polyhedron gene whose product represents 50% or more of total cellular proteins during a lytic infection. The baculovirus-expressed $p94^{RB}$ protein was subsequently purified by immunoaffinity chromatography.

3.3.1.3. Detection of $p94^{RB}$ Coding Expression Vectors

Expression vectors containing $p94^{RB}$ coding inserts can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a $p94^{RB}$ coding gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous/complementary to the inserted $p94^{RB}$ coding gene. Such hybridization can be carried out under stringent or nonstringent conditions, depending upon the size and sequence of the probe selected. In the second approach, the expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, viral occlusion formation in a baculovirus vector infected insect cell, etc.) caused by introduction of the expression vector into the host cell. For example, if the $p94^{RB}$ coding gene is inserted within a vector having a dominant selectable marker gene, such as a neomycin phosphotransferase gene under separate control of an appropriate promoter, such as an SV40 early promoter, the expression vector containing the $p94^{RB}$ coding gene can be identified by the presence of the marker gene function (geneticin resistance). In

the third approach, expression vectors containing a $p94^{RB}$ coding gene can be identified by assaying the $p94^{RB}$ coding gene products expressed by the vectors. Such assays can be based, for example, on the physical or functional properties of the $p94^{RB}$ gene products in *in vitro* or *in vivo* assay systems including metabolic radiolabelling by [^{35}S] methionine, SDS-polyacrylamide gel electrophoresis, binding with a specific antibody, and phosphorylation by a protein kinase.

3.3.2. Expression of $p94^{RB}$

An appropriate $p94^{RB}$ coding expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the $p94^{RB}$ protein-coding sequence may be introduced into a host cell. A host cell may be any cell type compatible with the vector for expressing and producing $p94^{RB}$. In a preferred embodiment, the host cell is a mammalian tumor cell to be treated. In a more preferred embodiment, the host cell is a human tumor cell to be treated. Expression of the $p94^{RB}$ in a host cell may be transient, permanent, or inducible.

The necessary transcriptional and translational signals, including promoter/enhancer sequences can also be supplied by the native RB gene and/or its flanking regions. A variety of vector/host systems may be utilized to express the $p94^{RB}$ protein-coding sequence in a tumor cell to be treated. These include but are not limited to mammalian cell systems transfected, infected or transduced with a plasmid, or a virus (e.g., adenovirus, retrovirus, herpes simplex virus, avipox virus). The expression elements of vectors vary in their strengths and specificities. Depending on the host cell to be treated, any one or more of a number of suitable transcription and translation elements may be used.

3.3.3. Methods of Treatment

The $p94^{RB}$ encoding gene construct of the present invention may be placed by methods well known to the art into an expression vector such as a plasmid or viral expression vector. A plasmid expression vector may be introduced into a tumor cell by calcium phosphate transfection, liposome (for example, LIPOFECTIN)-mediated transfection, DEAE Dextran-mediated transfection, polybrene-mediated transfection, electroporation and any other method of introducing DNA into a cell.

A viral expression vector may be introduced into a target cell in an expressible form by infection or transduction. Such a viral vector includes, but is not limited to: a retrovirus, an adenovirus, a herpes virus and an avipox virus. When $p94^{RB}$ is expressed in any abnormally proliferating cell, the cell replication cycle is arrested, thereby resulting in senescence and cell death and ultimately, reduction in the mass of the abnormal tissue, i.e., the tumor or cancer. A vector able to introduce the gene construct into a target cell and able to express $p94^{RB}$ therein in cell proliferation-suppressing amounts can be administered by any effective method.

For example, a physiologically appropriate solution containing an effective concentration of active vectors can be administered topically, intraocularly, parenterally, orally, intranasally, intravenously, intramuscularly, subcutaneously or by any other effective means. In particular, the vector may be directly injected into a target cancer or tumor tissue by a needle in amounts effective to treat the tumor cells of the target tissue.

Alternatively, a cancer or tumor present in a body cavity such as in the eye, gastrointestinal tract, genitourinary tract (e.g., the urinary bladder), pulmonary and bronchial system and the like

can receive a physiologically appropriate composition (e.g., a solution such as a saline or phosphate buffer, a suspension, or an emulsion, which is sterile except for the vector) containing an effective concentration of active vectors via direct injection with a needle or via a catheter or other delivery tube placed into the cancer or tumor afflicted hollow organ. Any effective imaging device such as X-ray, sonogram, or fiberoptic visualization system may be used to locate the target tissue and guide the needle or catheter tube.

In another alternative, a physiologically appropriate solution containing an effective concentration of active vectors can be administered systemically into the blood circulation to treat a cancer or tumor which cannot be directly reached or anatomically isolated.

In yet another alternative, target tumor or cancer cells can be treated by introducing $p94^{RB}$ protein into the cells by any known method. For example, liposomes are artificial membrane vesicles that are available to deliver drugs, proteins and plasmid vectors both in vitro or in vivo (Mannino, R.J. et al., 1988, Biotechniques, 6:682-690) into target cells (Newton, A.C. and Huestis, W.H., Biochemistry, 1988, 27:4655-4659; Tanswell, A.K. et al., 1990, Biochmica et Biophysica Acta, 1044:269-274; and Ceccoll, J. et al. Journal of Investigative Dermatology, 1989, 93:190-194). Thus, $p94^{RB}$ protein can be encapsulated at high efficiency with liposome vesicles and delivered into mammalian cells in vitro or in vivo.

Liposome-encapsulated $p94^{RB}$ protein may be administered topically, intraocularly, parenterally, intranasally, intratracheally, intrabronchially, intramuscularly, subcutaneously or by any other effective means at a dose efficacious to treat the

abnormally proliferating cells of the target tissue. The liposomes may be administered in any physiologically appropriate composition containing an effective concentration of encapsulated $p94^{RB}$ protein.

3.3.4. Tumors Susceptible To Treatment

The gene construct and vectors of the present invention are effective in inhibiting the growth or mitosis or both of any type of tumor cell. The gene construct of the invention has demonstrated effectiveness in treating tumor cells of carcinomas and sarcomas. In particular, the gene construct of the invention has demonstrated effectiveness in suppressing replication and inducing cell senescence followed by cell death in the following tumor cell types: bladder carcinoma, lung carcinoma, breast carcinoma, prostate carcinoma, fibrosarcoma, osteosarcoma and cervix carcinoma.

Further, the gene construct of the invention has demonstrated effectiveness in suppressing replication and inducing cell senescence followed by cell death in the tumor cells having the following identified genetic defects: tumor suppressor gene RB and p53 mutation, oncogene *myc* activation, and oncogene N-ras and c-*yes*-1 activation.

Furthermore, the gene construct of the invention has demonstrated effectiveness in suppressing replication and inducing cell senescence followed by cell death in the tumor cells having normal endogenous tumor suppressor RB^{110} and/or p53 gene expression.

In addition, the gene construct of the invention is able to suppress replication in lymphomas, leukemia and in tumor cells having tumor suppressor gene DCC and NF1 genetic defects, as well as in other tumor cell types in which the genetic defects are unknown or have yet to be identified.

3.3.5. Ex Vivo Treatment of Tumor or Cancer Tissues

In a preferred embodiment a tumor cell is transduced with a retrovirus vector, an adenovirus vector, a plasmid vector or any other appropriate vector capable of expressing the $p94^{RB}$ protein in that tumor cell. The cancer cell may be present in a blood or bone marrow sample collected from a leukemia patient. A dose of $p94^{RB}$ protein expressing retrovirus vector or adenovirus vector or plasmid vector or any other appropriate vector is administered to the sample of blood or bone marrow at a dose sufficient to transduce enough cells in the sample to produce a reduction in tumor cell numbers. The cell proliferation of the treated cancer cells will be slowed or terminated followed by a process similar to normal cellular differentiation or cell senescence. Analo-gously, blood or bone marrow or other tissue is treated ex vivo using an effective dose of a lipsome-encapsulated $p94^{RB}$ protein. Thereafter the sample may be returned to the donor or infused into another recipient.

3.3.6. In Vivo Treatment of Tumor or Cancer Tissues

Methods of administering viral vectors are well known. In general, the skilled artisan will appreciate that a retroviral vector, an adenovirus vector, a plasmid vector, or any other appropriate vector capable of expressing the $p94^{RB}$ protein can be administered in vivo to a cancer by a wide variety of manipulations. All such manipulations have in common the goal of placing the vector in sufficient contact with the target tumor to permit the vector to transduce or transfect the tumor cells. In a preferred embodiment, cancers present in the epithelial linings of hollow organs may be treated by

infusing the vector suspension into a hollow fluid filled organ, or by spraying or misting into a hollow air filled organ. Thus, the tumor cell may be present in or among the epithelial tissue in the lining of pulmonary bronchial tree, the lining of the gastrointestinal tract, the lining of the female reproductive tract, genito-urinary tract, bladder, the gall bladder and any other organ tissue accessible to contact with the vector.

In another preferred embodiment, the cancer may be located in or on the lining of the central nervous system, such as, for example, the spinal cord, spinal roots or brain, so that vectors infused in the cerebrospinal fluid will contact and transduce the cells of the tumor in that space.

In another preferred embodiment, the cancer is a solid tumor. The skilled artisan will appreciate that the vector can be administered to the tumor by direct injection of the vector suspension into the tumor so that vectors will contact and transduce or transfect the tumor cells inside the tumor.

In yet another preferred embodiment, the cancer may be a cancer of the blood, blood forming organs or any organ directly perfused by the blood, so that vectors injected into the blood stream will contact and treat the cells of the cancer. Thus, the cancer may be a leukemia, a lymphoma or other tumor type and the tumor cell may be present in the blood, the bone marrow, the spleen, the thymus, the liver and any other blood perfused organ.

The skilled artisan will understand that the vector is administered in a composition comprising the vector together with a carrier or vehicle suitable for maintaining the transduction or transfection efficiency of the chosen vector and promoting a safe infusion. Such a carrier may be a pH balanced physiological buffer, such as a phosphate, citrate or

bicarbonate buffer, a saline solution, a slow release composition and any other substance useful for safely and effectively placing the vector in contact with abnormally proliferating cells to be treated.

The invention is further described in the following examples which are in no way intended to limit the scope of the invention.

4. Examples

4.1 Preparation of Vectors for Expression of the Second In-Frame AUG Codon-Initiated RB Protein in Insect Cells

The engineered derivatives of Autographa california Multiple Nuclear Polyhedrosis Virus ("AcMNPV") have been widely employed to produce high levels of accurately processed and biologically active proteins. This baculovirus propagates in cultured Fall Army worm Spondoptera frugiperda cells (Sf9 cells) and has a strong temporarily regulated promoter of the polyhedron gene whose product represents 50% or more of total cellular proteins during a lytic infection.

By in vivo recombination, the coding sequence of a foreign gene can easily be placed under the transcriptional control of the polyhedron promoter, resulting in a high level of expression. In addition, such proteins may be correctly folded and contain appropriate post-translational modifications like those proteins in the native higher eukaryotes.

By site-specific mutagenesis, two BamH1 sites were introduced into the RB cDNA at nucleotides +7 and +3230 (the A of the second in-frame AUG codon is designated +19). The resulted DNA molecule has the nucleotide sequence of Figure 1 (SEQ ID NO:1; SEQ ID NO:2), which is also referred to herein as the second in-frame AUG codon-initiated RB protein gene, or the $p94^{RB}$ encoding gene. The coded-for protein has the

sequence of Figure 2 (SEQ ID NO:3) and is referred to herein as the second in-frame AUG codon-initiated RB protein, or the $p94^{RB}$ protein.

In an attempt to achieve maximal production of the second in-frame AUG codon-initiated RB protein in the baculovirus expression system, the recombinant transfer vector was constructed with insertion of the $p94^{RB}$ gene into the pVL1393 plasmid so that the $p94^{RB}$ gene was placed under the control of the polyhedron gene promoter.

As shown in Figure 3, the resulting pVL-s-RB plasmid contains no additional AUG start codon upstream from the $p94^{RB}$ translation initiation site at nucleotide +19, and thus encodes a nonfusion $p94^{RB}$ protein. In a parallel study, the same strategy was employed to construct a $p110^{RB}$ expression vector which was designated pVL/1st AUG-RB.

Transfer of RB cDNAs from the recombinant vectors to the viral genome was accomplished by co-transfecting wild-type AcMNPV virus DNA with pVL-s-RB plasmid DNA or pVL/1st AUG-RB plasmid DNA. The recombinant viruses were subjected to three rounds of plaque purification to obtain a pure stock of RB-containing baculovirus, designated AcMNPV-RB94 and AcMNPV-RB110, respectively.

4.2 Purification of $p110^{RB}$ and $p94^{RB}$ Proteins

The $p110^{RB}$ and $p94^{RB}$ proteins were purified from baculovirus-infected insect cells by immunoaffinity chromatography. Briefly, insect cells were harvested 24 hours after the virus infection and lysed at 4°C with EBC buffer (50 mM Tris-HCl, pH8.0, 120 mM NaCl, 0.5% NP-40, 50μg/ml aprotinin). The lysate was clarified by centrifugation and the $p110^{RB}$- or $p94^{RB}$-containing supernatant was incubated with biotinylated WL-1 polyclonal anti-RB antibodies (Xu, H-J., et al., 1989, Oncogene, 4:807-812) at 4°C overnight. The

procedures for biotinylation of rabbit IgGs using succinimide ester followed the methods described by Bayer and Wilchek (Baylor, E.A. and Wilchek, M., 1980, Methods Biochem. Anal., 26:1-45). The RB protein-IgG-biotin complex was collected on a streptavidin agarose gel column. Purified $p110^{RB}$ or $p94^{RB}$ were eluted from separate columns using 100 mM glycine (pH 2.2) and neutralized with 1M of phosphate (pH 8.0).

4.2.1. $p94^{RB}$ Shares Major Biochemical and Biological Properties With $p110^{RB}$

Since non-functional mutations of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association and nuclear localization (Templeton et al., 1991, Proc. Natl. Acad. Sci., USA, 88:3033-3037), the functional aspects of the artificial $p94^{RB}$ protein were studied for these characteristics.

First, to determine whether the RB proteins produced in the insect cells with the baculoviruses were associated with the nucleus, the AcMNPV-RB110 and AcMNPV-RB94 infected Sf9 cells were immunostained with MAb-1 anti-RB monoclonal antibody 24h after infection. As shown in Figure 4, intense staining was found exclusively in the nuclei of cells infected with either AcMNPV-RB110 (panel B) or AcMNPV-RB94 (panel C).

The $p110^{RB}$ and $p94^{RB}$ proteins purified from baculovirus-infected insect cells by immunoaffinity chromatography were tested for their ability to form a specific complex with SV40 T antigen. Briefly, equal amounts of $p94^{RB}$ or $p110^{RB}$ and T antigen were mixed and aliquots of the mixture were immunoprecipitated with PAB419 anti-T antibody. As shown in Figure 5, mixing of $p94^{RB}$ (or $p110^{RB}$) with T antigen in vitro resulted in the co-immunoprecipitation of both under- and hypo-phosphorylated $p94^{RB}$ (lane 5), or $p110^{RB}$ (lane 3) with

PAB419. The data demonstrated that either $p110^{RB}$ or $p94^{RB}$ protein can form a specific complex with SV40 T antigen. The AcMNPV-RB94 virus-infected insect cells appear to make hyperphosphorylated $p94^{RB}$ (lane 4), which was unable to form complexes with SV40 T antigen (compare lane 4 with lane 5).

The Western blot shown in Figure 5 revealed an apparent relative molecular mass (Mr) of 94 kD for the second in-frame AUG codon-initiated RB protein. On SDS-PAGE, the $p94^{RB}$ protein (Figure 5, lanes 4 and 5) was smaller than the naturally occurring 98 kDa proteins of unknown origin (Xu et al., 1989, Oncogene, 4:807-812) (Figure 5, lane 1). Therefore, the second in-frame AUG codon-initiated RB protein of this invention ($p94^{RB}$) has not been found to occur naturally in human cells.

It is concluded that the second in-frame AUG codon-initiated $p94^{RB}$ protein produced in recombinant virus-infected insect cells is a artificial but stable nuclear phosphoprotein with its under- and hypo-phosphorylated forms being able to assemble specific complex with SV40 T antigen, as does the naturally occurring RB protein species, $p110^{RB}$.

4.3 Construction of Expression Vectors for Mammalian Cells

4.3.1. Subcloning of RB cDNA Fragments Encoding for the First and Second In-Frame AUG Codon-Initiated RB Proteins

Subcloning of RB cDNA fragments encoding for the first and second in-frame AUG codon-Initiated RB proteins was accomplished by standard methods in the art. The methods for DNA manipulation were modified from Maniatis, T., Fritsch, E.F., and Sambrook, J. (1989): Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; and Ausubel, F.M., Brent, R., Kingston, R.E.,

Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1992): Current Protocols in Molecular Biology, John Wiley & Sons, New York.

4.3.2. Preparation of a DNA Molecule Encoding the Second In-Frame AUG Codon-Initiated RB Protein.

A plasmid, p4.95BT (Friend et al., 1987, Proc. Natl. Acad. Sci. USA, 84:9059-9063) or F7 (Takahashi, R., Hashimoto, T., Xu, H-J., et al., 1991, Proc. Natl. Acad. Sci. USA, 88:5257-5261) containing the full length retinoblastoma (RB) gene cDNA was digested with the restriction enzyme, Hind II, at nucleotide +7 and the restriction enzyme, ScaI, at nucleotide 3,230 (The A of the second in-frame AUG codon of the full length RB cDNA open reading frame was designated nucleotide +19). The resulted 3,230 bp RB cDNA fragment had two blunt ends. Conversion of the blunt ends to restriction enzyme BamHI sites was done by ligation of a synthetic BamHI oligonucleotide linker (GGGATCCC) to each blunt end of the fragment followed by digestion with the BamHI enzyme.

The desired RB cDNA fragment was inserted into the BamHI cloning site of a plasmid vector, pUC19, and propagated in the Escherichia coli strain, DH5 alpha bacterial cells. The recombinant plasmid was purified from a single DH5 alpha transformant and designated plasmid pUC-s-RB. This plasmid contains the desired RB cDNA fragment of 3,230 bp coding for the second in-frame AUG codon-initiated RB protein of 816 amino acids.

4.3.3. Preparation of A DNA Molecule Encoding the First In-Frame AUG Codon-Initiated RB Protein.

The full length RB cDNA plasmid was digested with the restriction enzyme, AcyI at nucleotide -322 and ScaI at nucleotide 3,230. The AcyI ends (overhang

5'-CG) were repaired by "filling in" the ends with the Klenow fragment of *E. coli* DNA polymerase I in the presence of all 4 dNTPs to generate blunt ends. Conversion of the blunt ends to restriction enzyme BamHI sites was done as described above. The resulted RB cDNA fragment of 3,552 bp was inserted into the plasmid pUC19 and propagated in the Escherichia coli strain DH5 alpha, which was subsequently purified from a single DH5 alpha transformant and designated plasmid pUC-f-RB. This plasmid contains the RB cDNA fragment of 3,552 bp coding for the first in-frame AUG codon-initiated RB protein of 928 amino acids.

4.3.4. Construction of $p94^{RB}$ Expression Plasmid Using A Human ß-Actin Gene Promoter

The RB cDNA fragment of 3,230 bp coding for the second in-frame AUG codon-initiated RB protein of 816 amino acids ($p94^{RB}$) was recovered from plasmid pUC-s-RB following the restriction enzyme, BamHI digestion, and re-inserted into the unique BamHI site of an expression plasmid, pHßAPr-1-neo (Gunning, P., et al., Proc. Natl. Acad. Sci., USA, 1987, 84:4831-4835) in a orientation that the $p94^{RB}$ coding sequence was under the direct control of the ß-actin gene promoter. A plasmid vector with the correct insert orientation was selected by restriction endonuclease mapping after propagation in DH5 alpha Escherichia coli host cells, and was designated pßA-s-RB34 (Figure 7B). The corresponding DH5 alpha strain that contains plasmids pßA-s-RB34 was thereafter designated DHB-s-RB34 (ATCC 69241, patent depository, American Type culture Collection).

The plasmid vector pßA-s-RB34 contains no additional AUG codon between the ß-actin gene promoter and the second in-frame AUG codon of the RB coding sequence, and thus encodes a non-fusion $p94^{RB}$ protein.

The plasmid vector pßA-s-RB34 also confers a dominant selectable marker (geneticin resistance) in eukaryotic cells through expression of the neomycin phosphotransferase (neo) under separate control of an SV40 early promoter (Figure 7, sv-neo).

4.3.5. Construction of $p110^{RB}$ Expression Plasmid Using A Human ß-Actin Gene Promoter

The RB cDNA fragment of 3,552 bp coding for the first in-frame AUG codon-initiated RB protein of 928 amino acids ($p110^{RB}$) was recovered from plasmid pUC-f-RB and re-inserted into the expression plasmid pHßAPr-1-neo downstream from the ß-actin gene promoter. The resulting plasmid vector was designated pßA-f-RB33 (Figure 7A). The plasmid vector pßA-f-RB33 contains no additional AUG codon between the ß-actin gene promoter and the first in-frame AUG codon of the RB coding sequence, and thus encodes a non-fusion $p110^{RB}$ protein.

4.3.6. Construction of $p94^{RB}$ and $p110^{RB}$ Expression Plasmids Using A Cytomegalovirus Promoter (CMVp)

Alternatively, an expression plasmid, pCMV-Neo-Bam (Baker, S.J., et al., Science, 1990, 249:912-915) was used in place of plasmid pHßAPr-1-neo. The vector included cytomegalovirus (CMV) promoter/enhancer sequences, which could drive expression of the insert at the BamHI site, and splicing and polyadenylation sites derived from the rabbit ß-globin gene, which ensured proper processing of the transcribed insert in the cells. A pBR322 origin of replication and ß-lactamase gene facilitated propagation of the plasmid in *E. coli*. The plasmid conferred geneticin resistance (a selectable marker in eukaryotic cells)

through expression of the neomycin phosphotransferase (neo) under the control of a herpes simplex virus (HSV) thymidine kinase promoter.

The same strategies as described *supra* in Sections 4.3.4 and 4.3.5 were employed to transfer the RB cDNA fragments of 3,230 bp and 3,552 bp from plasmids pUC-s-RB and pUC-f-RB, respectively, to the unique BamHI site in the expression vector, pCMV-Neo-Bam. The resulting plasmid vectors were designated by the names of pCMV-s-RB42, expressing the $p94^{RB}$ and pCMV-f-RB35, expressing the $p110^{RB}$ (Figure 6). The corresponding Escherichia coli DH5 alpha strain which contains plasmids pCMV-s-RB42 was thereafter designated DHC-s-RB42 (ATCC 69240, patent depository, American Type Culture Collection).

4.3.7. Construction of $p94^{RB}$ Protein Expression Retrovirus Vectors

For this protocol, retroviral vector, pLLRNL (Miller, A.D., Law, M.-F., Verma, I.M., Molec. Cell Biol., 1985, 5:431) and amphotropic retrovirus packaging cell line, PA317 (ATCC CRL9078) (Miller, A.D., and Buttimore, C., Molec. Cell Biol., 1986, 6:2895-2902) are used.

A plasmid p4.95BT or F7 containing the full-length RB gene cDNA is digested with the restriction enzyme Hind II at nucleotide +7 (the A of the second in-frame AUG codon of the full-length RB cDNA open reading frame was designated nucleotide +19). Conversion of the Hind II site to restriction enzyme Hind III site is done by ligation of a synthetic Hind III oligonucleotide linker (CCAAGCTTGG) to the blunt ends of the linear plasmid DNA, followed by digestion with the Hind III enzyme. The linear plasmid DNA is further digested with restriction enzyme, ScaI, at nucleotide 3,230. The resulted RB cDNA fragment of 3,230 bp codes for the second

in-frame AUG codon-initiated RB protein of 816 amino acids ($p94^{RB}$). This fragment has a 5'-Hind III site (cohesive end) and a 3'-ScaI site (blunt end), which facilitates its insertion into the retroviral vector, pLLRNL.

The vector pLLRNL is digested with two sets of restriction enzymes: Hind III/ClaI and SmaI/ClaI to delete the luciferase gene. Appropriate fragments are recovered from the agarose gel following electrophoresis, and ligated with the RB cDNA fragment of 3,230 bp to form a new vector, pLRB94RNL, in which the $p94^{RB}$ expression is under the control of the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTRs).

The basic protocol for construction of the retroviral vector, pLRB94RNL, is modified from Huang, H.-J.S., et al., 1988, Science, 242:1563-1566.

Alternatively, the vector pLLRNL is digested with a single restriction enzyme, Hind III, to delete the luciferase gene, as well as the Rous sarcoma virus promoter (RSV). An appropriate DNA fragment is recovered from the $p94^{RB}$ expression plasmid, pCMV-s-RB42 (or pβA-s-RB34). The recovered DNA fragment, which contains the 3,230 bp RB cDNA fragment and 5'-flanking CMV promoter (or ß-actin promoter), is inserted into the ClaI restriction site of the retroviral vector. Conversion between the restriction enzyme sites is done by the methods as described *supra* in Section 4.3.7.

In the resulting $p94^{RB}$ expression retrovirus vector, the $p94^{RB}$ gene is under the control of an internal promoter (the CMV promoter or ß-actin promoter), while the Tn5 neomycin-resistance gene (Neo) is under the control of the MuLV LTRs.

A safe and efficient amphotropic packaging cell line is necessary for transfer of retroviral vector genes into human cancer cells. The virus packaging

methods are modified from the method of Miyanohara et al., Proc. Natl. Acad. Sci., USA, 1988, 85:6538-6542. For this protocol, the PA317 packaging cell line is used. This packaging cell line has received prior approval for use in human gene therapy clinical trials.

The retroviral vector (pLRB94RNL) DNA is transfected into PA317 packaging cells by LIPOFECTIN reagent (GIBCO BRL Life Technologies, Inc., Gaithersburg, MD) or electroporation methods as described in Sections 4.4.1. *infra*. Single colonies are isolated by selection in G418-containing medium (400 μg/ml) and expanded into mass cultures. To titer the virus produced by selected PA317 clones, dilutions of cell-free culture medium from each PA317 clone are applied to 208F rat fibroblasts (indicator cells) in the presence of POLYBRENE (Sigma, 4 μg/ml) and G418 selection (400 μg/ml) is started 24 hours after infection.

After two weeks, G418-resistant colonies are visualized by Giemsa staining and viral titers are determined (colony-forming units per milliliter, cfu/ml). PA317 clones producing high virus titers are then assayed for human $p94^{RB}$ protein expression by Western immunoblotting as described previously (Xu, H.-J., et al., Oncogene, 1991, 6:1139-1146). Cell-free culture media from selected PA317 clones expressing high level of human $p94^{RB}$ protein are then applied to human cancer cells ex vivo or in vivo.

4.3.8. Construction of $p94^{RB}$ Protein Expression Adenovirus Vectors

The recombinant adenovirus Ad-RB94 is constructed from the adenovirus type 5 (Ad5) deletion mutant, Ad-dl324, and a plasmid, pTG5955, in which the human CFTR cDNA has been replaced by the human RB cDNA fragment of 3,230 bp coding for the $p94^{RB}$ protein. The

plasmid pTG5955 containing the RB cDNA insert is linearized by restriction enzyme ClaI cleavage and co-transfected with the large fragment of ClaI-cut Ad-dl324 DNA into 293 (ATCC CRL1573) cells to allow homologous recombination to occur, followed by replication and encapsidation of recombinant adenoviral DNA into infectious virions and the formation of plaques. Individual plaques are isolated and amplified in 293 cells, viral DNA is isolated and recombinant adenovirus plaques containing the human RB cDNA (Ad-RB94) are identified by restriction cleavage and Southern analysis. Ad-RB94 viruses are propagated in 293 cells and recovered 36 hours after infection. The viral preparation is purified by CsCl density centrifugation, and stored in virus dialysis buffer (10 mM Tris-Hcl, pH7.4; 1 mM $MgCl_2$) at 4°C for immediate use; or stored at -70°C prior to use (with the addition of 10% glycerol). The basic protocol for construction of the recombinant adenovirus Ad-RB94 is modified from Rosenfeld, M.A., et al., Cell, 1992, 68:143-155.

4.3.9. Physical DNA Transfer Method

An alternative gene transfer method that has been approved for use in humans by the Food and Drug Administration is the transfer of plasmid DNA in liposomes directly to tumor cells in situ (Nabel, E.G., et al., 1990, Science, 249:1285-1288). Plasmid DNA is easy to certify for use in humans because, unlike retroviral vector, it can be purified to homogeneity.

The $p94^{RB}$ expressing plasmid vectors pCMV-s-RB42 or pβA-s-RB34 are used to form complexes with liposomes, and directly treat tumor cells in vivo (or ex vivo). In this procedure, as described in Section 4.4.1 *infra*, stable integration of the DNA

into transfected tumor cells is not required for gene therapy as transient expression may suffice.

4.4. Treatment of Human Tumor Cells In Vitro With $p94^{RB}$ Plasmid Vectors pβA-s-RB34 or pCMV-s-RB42.

4.4.1. Treatment of RB-Defective Human Tumor Cells In Vitro

Human tumor cells having known RB gene deficiencies were treated with the $p94^{RB}$ plasmid vector pβA-s-RB34 (or pCMV-s-RB42). These include: 1) human bladder carcinoma cell line, 5637, (ATCC HTB9); 2) human breast carcinoma cell line, MDA-MB-468 (ATCC HTB132); 3) human non-small cell lung carcinoma cell line, H2009 (Kratzke, R.A., et al., 1992, The Journal of Biological Chemistry, 267:25998-26003); 4) human prostate carcinoma cell line, DU145 (ATCC HTB81); 5) human osteosarcoma cell line, Saos2 (ATCC HTB85); and 6) human fibrosarcoma metastatic to lung cell line, Hs913T (ATCC HTB152).

For treatment, tumor cells were transiently transfected with the plasmid DNA pβA-s-RB34 (or pCMV-s-RB42) via LIPOFECTIN reagent (GIBCO BRL Life Technologies, Inc. Gaithersberg, MD). Similar results have been obtained from transfection using calcium phosphate or electroporation methods.

The following procedures for transfection using LIPOFECTIN were modified from the manufacturer's specifications. Tumor cells were seeded in 100-mm dishes in appropriate growth medium supplemented with serum. The cells were incubated at 37°C in a 5% CO_2 environment until the cells were 40-60% confluent. This usually took 18-24 hours, but the time varied among cell types. The following solution was prepared in 17 x 75 mm polystyrene tubes: Solution A - for each dish of cells to be transfected, 5-10μg of plasmid DNA were diluted into a final volume of 100μl

with serum-free medium; Solution B - for each dish of cells to be transfected, 30-50μl of LIPOFECTIN reagent was diluted into a final volume of 100μl with serum-free medium. The two solutions were combined, mixed gently, and incubated at room temperature for 10-15 min. The LIPOFECTIN reagent interacted spontaneously with plasmid DNA to form a lipid-DNA complex. While the lipid-DNA complex was forming, the cells were washed twice with 6 ml of serum-free medium. For each transfection, 6 ml of serum-free medium were added to each polystyrene tube containing the lipid-DNA complex. The solution was mixed gently, and the medium-complex was overlayed onto the cells. The dishes were then swirled gently to ensure uniform distribution. The dishes were then incubated at 37°C in a 5% CO_2 incubator. After 12 to 24 hours the medium-complex was replaced with appropriate growth medium containing 10% fetal calf serum.

In parallel studies, tumor cells were transfected with the plasmid DNA pβA-f-RB33 or pCMV-f-RB35 which expresses the $p110^{RB}$. The following assays were used to evaluate the growth inhibitory effects of introducing $p94^{RB}$ versus $p110^{RB}$ expression in RB defective tumor cells:

1) DNA synthesis in tumor cells treated with plasmid vectors.

After plasmid DNA treatment the tumor cells were labeled with [^{3}H]-thymidine for 2 hours, then transferred to polylysine-coated slides, fixed and immunocytochemically stained with a monoclonal anti-RB antibody, MAb-1 (Triton Biosciences, Inc. Alameda, CA). The RB-positive transfected cells were counted under the microscope. The slides were then coated with Kodak NTB2 autoradiographic emulsion and exposed for 7-10 days. The [^{3}H]-thymidine labeling and RB protein immunocytochemical staining were done

according to the methods previously described (Xu et al., Oncogene, 1991, 6:1139-1146). About 400 to 1600 RB-positive and 600 RB-negative tumor cells were assessed for each determination of [^{3}H]-thymidine uptake. The study demonstrated that the RB-defective tumor cells expressing p94^{RB} did not progress through the cell cycle, as evidenced by their failure to incorporate [^{3}H]-thymidine into DNA (Table 2). However, the percentage of cells undergoing DNA replication was only slightly lower in cells producing p110^{RB} than in cells that were RB-negative (Table 2).

Table 2. Immunocytochemical Staining and [^{3}H] Thymidine Incorporation of RB-Defective Tumor Cells Following Transfection With p94^{RB} or p110^{RB} Expression Plasmids

Recipient Cells	Promoter	Protein Expressed	Cells Incorporating [3H] Thymidine RB+	RB-
5637	ß-actin gene promoter	p110^{RB}	34%	45%
		p94^{RB}	2.3%	43%
	Cytomegalovirus promoter/enhancer	p110^{RB}	21%	—
		p94^{RB}	1.8%	---
MDA-MB-468	Cytomegalovirus promoter/enhancer	p110^{RB}	14%	40%
		p94^{RB}	0.5%	39%
H2009	β-actin gene promoter	p110^{RB}	19%	26%
		p94^{RB}	0.1%	27%
DU145	Cytomegalovirus Promoter/enhancer	p110^{RB}	23%	33%
		p94^{RB}	1.0%	33%
Hs913T	Cytomegalovirus promoter/enhancer	p110^{RB}	18%	34%
		p94^{RB}	0.9%	36%
Saos2	Cytomegalovirus promoter/enhancer	p110^{RB}	19%	32%
		p94^{RB}	0.9%	35%

2) Colony formation assay.

Approximately 48 hours after transfection the tumor cells were replated at a density of 10^5 cells per 100 mm dish with selected medium containing G418 of 400-600 μg/ml. Cells were cultured for 2 to 3 weeks

and colonies of >100 cells were scored. The data are illustrated in Table 3. Cells treated with plasmid vectors expressing $p94^{RB}$ formed approximately four-fold fewer colonies than those transfected with $p110^{RB}$ plasmid vectors. The difference was statistically significant ($p < 0.05$ by t-test).

Furthermore, in those colonies that did form after $p94^{RB}$ plasmid DNA treatment, $p94^{RB}$ protein expression was no longer observed. Failure to isolate long-term cultures expressing the $p94^{RB}$ protein in treated tumor cells shows that $p94^{RB}$ did suppress tumor cell growth. In contrast, 7 of 48 cell lines (approximately 15%) derived from tumor cells after transfection with the $p110^{RB}$ plasmid DNA were found to express the $p110^{RB}$ protein. This percentage was consistent with results expected in human cells transfected with a vector containing two independent transcription units and therefore introduction of $p110^{RB}$ expression does not exert growth inhibitory effects on RB-defective tumor cells.

Table 3: Growth inhibitory effects of introducing $p110^{RB}$ and $p94^{RB}$ expression into RB-defective bladder carcinoma cell line, 5637 (HTB9). Each number represents 6 to 11 dishes.

Vector Type	Number of G418-Resistant Colonies Formed		
	Vector	$p110^{RB}$	$p94^{RB}$
pCMV-Neo-Bam	280	24	6
pHßAPr-1-neo	—	33	8

3) The Effect of $p94^{RB}$ Expression on Cellular Morphology

The HTB9 transfectants were also immunostained with MAb-1 anti-RB monoclonal antibody about 24 hours after transfection. The staining results are illustrated in Figure 8.

As demonstrated in Figure 8, the majority of RB-positive, $p94^{RB}$-expressing HTB9 cells become very large in size, with lower nucleocytoplasmic ratio, or higher incidence of being multinucleated cells (panel C), a morphological change frequently associated with cellular senescence. However, such a morphological change has not been seen in group A, mock-transfected HTB9 cells and group B, $p110^{RB}$ expressing RB-positive HTB9 cells (Figure 8, panels A and B).

4.4.2. Treatment of Human Tumor Cells Having Normal ($p110^{RB}$) RB Expression (RB+)

Two RB+ human cell lines (i.e., having no RB gene defect), including a human fibrosarcoma cell line, HT1080 (ATCC CCL121), and human cervix carcinoma cell line, HeLa (ATCC CCL2) were treated with the $p94^{RB}$ protein expression plasmid, pCMV-s-RB42, using the LIPOFECTIN reagent as described *supra*. In parallel studies, these cell lines were also transfected with the $p110^{RB}$ protein expression plasmid, pCMV-f-RB35. The colony formation assay as described *supra* was used to evaluate the growth inhibitory effects of introducing exogenous $p94^{RB}$ versus $p110^{RB}$ expression in RB^+ tumor cells. As shown in Table 4, expression of the $p94^{RB}$ protein dramatically inhibited the cell growth of HT1080 and HeLa cells. There was a two- to nine-fold reduction in the number of G418-resistant colonies formed after treated with the plasmid victor pCMV-s-RB42 expressing $p94^{RB}$, while no such effect was observed by transfection with the pCMV-f-RB35 plasmid (expressing $p110^{RB}$ protein). The difference was statistically significant (the two-tailed P values were less than 0.03 as calculated by the paired t-test).

Table 4: Growth inhibitory effects of introducing $p110^{RB}$ and $p94^{RB}$ expression into RB-positive human fibrosarcoma cell line, HT1080 and the RB positive human cervix carcinoma cell line, HeLa. The RB expression was under the control of cytomegalovirus (CMV) promoter. For each experiment, three 5-cm^2 dishes were transfected and the total colonies counted after ten days of selection in G418 (1 mg/ml).

		No. of G418-Resistant Colonies Formed		
Recipient Cells	Experiment	Vector	$p110^{RB}$	$p94^{RB}$
HT1080	1	94	129	14
	2	88	122	16
	3	100	120	17
	4	99	110	15
HeLa	1	24	20	10
	2	25	24	9

4.5 Half-Life and Phosphorylation state of the $p94^{RB}$ Protein In Host Cells: The Distinct Properties of $p94^{RB}$

The half-life of transiently expressed $p94^{RB}$ and $p110^{RB}$ proteins in transfected bladder carcinoma cell line, 5637 (ATCC HTB9) was measured by pulse-labeling of transfected 5637 cells with [^{35}S]-methionine followed by a chase with excess unlabeled methionine (Figure 9).

The bladder tumor cells were transfected in multiple dishes with either $p110^{RB}$ (Figure 9, left) or $p94^{RB}$ (Figure 9, right) expression plasmids. Twenty-four hours after transfection the cells were labeled with [^{35}S]-methionine and chased with excess unlabeled methionine for 0, 6, 12 and 24 hours, respectively. RB proteins were determined by immunoprecipitation.

The half-life of $p94^{RB}$ protein in the transfected 5637 cells was determined to be 12 hours. In

contrast, the half-life of $p110^{RB}$ protein was 4-6 hours. Therefore, $p94^{RB}$ protein expressed in host tumor cells has a slower turnover, which is believed to contribute to its efficacy as a suppressor of both RB+ and RB- tumor cell replication.

The comparative phosphorylation states of $p110^{RB}$ and $p94^{RB}$ in transiently transfected 5637 cells were determined by Western blot analysis: cell-lysates were made from WI-38, parental 5637 and pβA-f-RB33 (expressing $p110^{RB}$, Section 4.3.5) or pβA-s-RB34 (expressing $p94^{RB}$, Section 4.3.4) plasmid transfected 5637 cells approximately 24 hours after transfection. The basic protocal for Western blot analysis was described in Xu, H-J., et al., 1989, Oncogene, 4:807-812. Each lane was loaded with 40μl of the lysate corresponding to 4 x 10^5 cultured cells. Proteins were separated by 8% SDS-PAGE and electroblotted to a PVDF membrane. After blocking with 3% non-fat milk in TBST (10mM Tris-HCl, pH8.0, 150mM NaCl, 0.05% Tween 20), the membrane was incubated with MAb-1 monoclonal anti-RB antibody at 0.1 $\mu g/cm^2$ overnight. The blot was then probed by the Enhanced Chemiluminescence (ECL) (Amersham Corporation, Arlington Heights, Illinois) immunodetection method. X-ray films were exposed for 2 seconds (Figure 10, lane 1) or 30 seconds (Figure 10, lanes 2-4).

Of particular interest was the fact that the RB-defective bladder carcinoma cell line, 5637, failed to phosphorylate the $p94^{RB}$ protein as shown by Western blot analysis (Figure 10, lane 4), although the $p110^{RB}$ proteins expressed in transfected 5637 cells were fully phosphorylated (Figure 10, lane 3). Therefore, the presence of only unphosphorylated $p94^{RB}$ proteins may also account for the failure of transfected 5637 tumor cells to enter S phase, and this in turn may cause cellular senescence and cell death.

4.6. Treatment of Human Bladder Cancers In Vivo.

The human bladder cancer represents an ideal model for practicing tumor suppressor gene therapy of solid tumors by infusing the $p94^{RB}$ protein expression retroviral vectors into the bladder. The original experimental model of human bladder cancer was established by Dr. Peter A. Jones and his colleagues (Ahlering, T.E., et al., Cancer Res., 1987, 47:6660-6665). It has been shown that human bladder tumor cells of RT4 cell line established from a superficial papillary tumor (which usually does not metastasize) produced tumors only locally when injected by a 22-gauge catheter into the bladder of female nude mice. In contrast, the EJ bladder carcinoma cells which were originally isolated from a more aggressive human bladder cancer produced invasive tumors in the nude mouse bladders which metastasized to the lung spontaneously (Ahlering, T.E., et al., Cancer Res., 1987, 47:6660-6665). Therefore, this model can be used for treatment of experimental bladder cancer by in vivo gene transfer with retroviral vectors.

Tumor cells from RB minus human bladder carcinoma cell line, 5637 (ATCC HTB9) and RB^+ human bladder carcinoma cell line, SCaBER (ATCC HTB3) are injected directly into the bladders of female athymic (nu/nu) nude mice (6 to 8 weeks of age) by a catheter as initially reported by Jones and his colleagues (Ahlering, T.E., et al., Cancer Res., 1987, 47:6660-6665).

Development and progression of the nude mouse bladder tumors are monitored using a fiber-optical system to which a TV monitor is attached. The experimental tumors are subsequently treated with retrovirus vectors expressing the $p94^{RB}$.

Supernatants with high virus titers are obtained from tissue culture media of selected PA317 clones

expressing high level of human $p94^{RB}$ protein (Section 4.3.7) and confirmed as free of replication-competent virus prior to use. The retroviral vector suspension at high titers ranging from 4×10^4 to greater than 1×10^7 colony-forming unit (cfu)/ml, and more preferably at a titer greater than 1×10^6 cfu/ml is then infused directly into the mouse bladders via a catheter to treat the tumors. The skilled artisan will understand that such treatments can be repeated as many times as necessary via a catheter inserted into the bladder. The tumor regression following transferring the $p94^{RB}$ gene is monitored frequently via the fiber-optic system mentioned above.

The same procedure as described above is used for treating the human bladder cancer except that the retroviral vector suspension is infused into a human bladder bearing cancer.

4.7. *In Vivo* Studies Using an Orthotopic Lung Cancer Model

Human large cell lung carcinoma, NCI-H460 (ATCC HTB177) cells which have normal $p110^{RB}$ expression are injected into the right mainstream bronchus of athymic (nu/nu) nude mice (10^5 cells per mouse). Three days later the mice are inoculated endobronchically with supernatant from the $p94^{RB}$, or $p110^{RB}$ retrovirus producer cells daily for three consecutive days. Tumor formation is suppressed in the group of mice treated with the $p94^{RB}$ retrovirus supernatant. In contrast, in the other group, which is treated with $p110^{RB}$ retrovirus supernatant, the majority of mice develop endobronchial tumors. This indicates that the $p94^{RB}$-expressing retrovirus inhibits growth of RB+ non-small cell lung carcinoma (NSCLC) cells, whereas the $p110^{RB}$-expressing retrovirus does not.

4.8. Treatment of Human Non-Small Cell Lung Cancers *In Vivo*.

Non-small cell lung cancer patients having an endobronchial tumor accessible to a bronchoscope, and also having a bronchial obstruction, are initially selected for $p94^{RB}$ gene therapy. Treatment is administered by bronchoscopy under topical or general anesthesia. To begin the procedure, as much gross tumor as possible is resected endoscopically. A transbronchial aspiration needle (21G) is passed through the biopsy channel of the bronchoscope.

The residual tumor site is injected with the appropriate retroviral vector supernatant (Section 4.3.7), adenovirus Ad-RB94 suspension (Section 4.3.8) or $p94^{RB}$-expressing plasmid vector-liposome complexes (Section 4.3.4 and 4.3.6) at a volume of 5 ml to 10 ml. Protamine is added at a concentration of 5 μg/ml. The injections of therapeutic viral or plasmid supernatant comprising one or more of the vectors are administered around and within the tumor or tumors and into the submucosa adjacent to the tumor. The injections are repeated daily for five consecutive days and monthly therafter. The treatment may be continued as long as there is no tumor progression. After one year the patients are evaluated to deterime whether it is appropriate to continue therapy.

In addition, as a precaution the patients wear a surgical mask for 24 hours following injection of the viral supernatant. All medical personnel wear masks routinely during bronchoscopy and injection of the viral supernatant. Anti-tussive is prescribed as necessary.

4.9 Treatment or Prevention of Human Lung Carcinomas With Liposome-Encapsulated Purified $p94^{RB}$ Protein

In yet another alternative, target tumor or cancer cells are treated by introducing $p94^{RB}$ protein into cells in need of such treatment by any known method. For example, liposomes are artificial membrane vesicles that have been extensively studied for their usefulness as delivery vehicles of drugs, proteins and plasmid vectors both in vitro or in vivo (Mannino, R.J. et al., 1988, Biotechniques, 6:682-690). Proteins such as erythrocyte anion transporter (Newton, A.C. and Huestis, W.H., Biochemistry, 1988, 27:4655-4659), superoxide dismutase and catalase (Tanswell, A.K. et al., 1990, Biochmica et Biophysica Acta, 1044:269-274), and UV-DNA repair enzyme (Ceccoll, J. et al. Journal of Investigative Dermatology, 1989, 93:190-194) have been encapsulated at high efficiency with liposome vesicles and delivered into mammalian cells in vitro or in vivo.

Further, small-particle aerosols provide a method for the delivery of drugs for treatment of respiratory diseases. For example, it has been reported that drugs can be administered in small-particle aerosols by using liposomes as a vehicle. Administered via aerosols, the drugs are deposited rather uniformly on the surface of the nasopharynx, the traceheobronchial tree and in the pulmonary area (Knight, V. and Gilbert, B., 1988, European Journal of Clinical Microbiology and Infectious Diseases, 7:721-731).

To treat or prevent lung cancers, the therapeutic $p94^{RB}$ protein is purified, for example, from recombinant baculovirus AcMNPV-RB94 infected insect cells by immunoaffinity chromatography (Sections 4.1 and 4.2) or any other convenient source. The $p94^{RB}$ protein is mixed with liposomes and incorporated into the liposome vesicles at high efficiency. The encapsulated $p94^{RB}$ is active. Since the aerosol delivery method is mild and well-tolerated by normal volunteers and patients, the $p94^{RB}$-containing liposomes

can be administered to treat patients suffering from lung cancers of any stage and/or to prevent lung cancers in high-risk population. The $p94^{RB}$ protein-containing liposomes are administered by nasal inhalation or by a endotracheal tube via small-particle aerosols at a dose sufficient to suppress abnormal cell proliferation. Aerosolization treatments are administered to a patient for 30 minutes, three times daily for two weeks, with repetition as needed. The $p94^{RB}$ protein is thereby delivered throughout the respiratory tract and the pulmonary area. The treatment may be continued as long as necessary. After one year the patent's overall condition will be evaluated to determine if continued therapy is appropriate.

4.10 $p94^{RB}$ Treatment is Non-Toxic to Normal Cells *In Vitro*

The retroviral vector, pLRB94RNL, expressing $p94^{RB}$ protein as described *supra* in section 4.3.7. was introduced into normal mouse fibroblast-derived retrovirus-packaging cell line, PA317 (ATCC CRL9078) by LIPOFECTIN reagent (GIBCO BRL Life Technologies, Inc., Gaithersburg, MD). Single cell colonies were isolated by selection in G418-containing medium and expanded into mass cultures. These clonal cells had been maintained over a one-year period of continuous culture, and stably expressed high levels of $p94^{RB}$ protein as determined by immunocytochemical staining (Figure 11) or by Western immunoblotting. These clones were indistinguishable from their parental normal mouse PA317 cells, or PA317 cells expressing human $p110^{RB}$ protein in terms of morphology (Figure 11) and growth rate. The results indicate that $p94^{RB}$ protein expression was non-toxic to normal cells *in vitro*.

4.11 $p94^{RB}$ Treatment is Non-Toxic to Normal Tissues *In Vivo*

The retroviral plasmid vector, pLRB94RNL, expressing $p94^{RB}$ protein as described *supra* in section 4.3.7. was mixed with DMRIE/DOPE Liposomes (VICAL, Inc., San Diego, CA) and infused directly into the mouse urinary bladders via a catheter. Forty-eight hours after treatment, the mice were sacrificed and bladders excised. As demonstrated by immunohistochemical staining of the $p94^{RB}$ protein in paraffin-embedded tissue sections from the mouse bladders (Figure 12), the liposome-encapsulated $p94^{RB}$ expressing retroviral plasmid vectors penetrated the mucosa of mouse bladders and expressed $p94^{RB}$ protein in the great majority of the transitional cells. The transitional epithelia expressing the $p94^{RB}$ were histologically normal (Figure 12, panel D, arrows), and were indistinguishable from the mucosa in untreated mouse bladders or mouse bladders treated with liposomes only (Figure 12). The results from such animal experiments strongly suggest that the $p94^{RB}$ treatment, unlike the conventional cytotoxic cancer therapy, is non-toxic to normal tissues *in vivo*.

5. Deposit of Microorganisms

The following were deposited on February 10, 1993 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852:

Escherichia coli DH5α	ATCC Designation
DHC-S-RB42	69240
DHB-S-RB34	69241

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such

modifications are intended to fall within the scope of the claims. Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Xu, Hong-Ji
Hu, Shi-Xue
Benedict, William F.

(ii) TITLE OF INVENTION: BROAD SPECTRUM TUMOR SUPPRESSOR GENES, GENE PRODUCTS, AND METHODS FOR TUMOR SUPPRESSION GENE THERAPY

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Pennie & Edmonds
(B) STREET: 1155 Avenue of the Americas
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: U.S.A.
(F) ZIP: 10036-2711

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: To be assigned
(B) FILING DATE: On even date herewith
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3232 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 19..2469

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GATCCCGACC TAGATGAG ATG TCG TTC ACT TTT ACT GAG CTA CAG AAA AAC        51
                    Met Ser Phe Thr Phe Thr Glu Leu Gln Lys Asn
                      1               5                  10
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ATA GAA ATC AGT GTC CAT AAA TTC TTT AAC TTA CTA AAA GAA ATT GAT          99
Ile Glu Ile Ser Val His Lys Phe Phe Asn Leu Leu Lys Glu Ile Asp
                15                  20                  25

ACC AGT ACC AAA GTT GAT AAT GCT ATG TCA AGA CTG TTG AAG AAG TAT         147
Thr Ser Thr Lys Val Asp Asn Ala Met Ser Arg Leu Leu Lys Lys Tyr
            30                  35                  40

GAT GTA TTG TTT GCA CTC TTC AGC AAA TTG GAA AGG ACA TGT GAA CTT         195
Asp Val Leu Phe Ala Leu Phe Ser Lys Leu Glu Arg Thr Cys Glu Leu
        45                  50                  55

ATA TAT TTG ACA CAA CCC AGC AGT TCG ATA TCT ACT GAA ATA AAT TCT         243
Ile Tyr Leu Thr Gln Pro Ser Ser Ser Ile Ser Thr Glu Ile Asn Ser
    60                  65                  70                  75

GCA TTG GTG CTA AAA GTT TCT TGG ATC ACA TTT TTA TTA GCT AAA GGG         291
Ala Leu Val Leu Lys Val Ser Trp Ile Thr Phe Leu Leu Ala Lys Gly
                80                  85                  90

GAA GTA TTA CAA ATG GAA GAT GAT CTG GTG ATT TCA TTT CAG TTA ATG         339
Glu Val Leu Gln Met Glu Asp Asp Leu Val Ile Ser Phe Gln Leu Met
            95                  100                 105

CTA TGT GTC CTT GAC TAT TTT ATT AAA CTC TCA CCT CCC ATG TTG CTC         387
Leu Cys Val Leu Asp Tyr Phe Ile Lys Leu Ser Pro Pro Met Leu Leu
        110                 115                 120

AAA GAA CCA TAT AAA ACA GCT GTT ATA CCC ATT AAT GGT TCA CCT CGA         435
Lys Glu Pro Tyr Lys Thr Ala Val Ile Pro Ile Asn Gly Ser Pro Arg
    125                 130                 135

ACA CCC AGG CGA GGT CAG AAC AGG AGT GCA CGG ATA GCA AAA CAA CTA         483
Thr Pro Arg Arg Gly Gln Asn Arg Ser Ala Arg Ile Ala Lys Gln Leu
140                 145                 150                 155

GAA AAT GAT ACA AGA ATT ATT GAA GTT CTC TGT AAA GAA CAT GAA TGT         531
Glu Asn Asp Thr Arg Ile Ile Glu Val Leu Cys Lys Glu His Glu Cys
                160                 165                 170

AAT ATA GAT GAG GTG AAA AAT GTT TAT TTC AAA AAT TTT ATA CCT TTT         579
Asn Ile Asp Glu Val Lys Asn Val Tyr Phe Lys Asn Phe Ile Pro Phe
            175                 180                 185

ATG AAT TCT CTT GGA CTT GTA ACA TCT AAT GGA CTT CCA GAG GTT GAA         627
Met Asn Ser Leu Gly Leu Val Thr Ser Asn Gly Leu Pro Glu Val Glu
        190                 195                 200

AAT CTT TCT AAA CGA TAC GAA GAA ATT TAT CTT AAA AAT AAA GAT CTA         675
Asn Leu Ser Lys Arg Tyr Glu Glu Ile Tyr Leu Lys Asn Lys Asp Leu
    205                 210                 215

GAT GCA AGA TTA TTT TTG GAT CAT GAT AAA ACT CTT CAG ACT GAT TCT         723
Asp Ala Arg Leu Phe Leu Asp His Asp Lys Thr Leu Gln Thr Asp Ser
220                 225                 230                 235

ATA GAC AGT TTT GAA ACA CAG AGA ACA CCA CGA AAA AGT AAC CTT GAT         771
Ile Asp Ser Phe Glu Thr Gln Arg Thr Pro Arg Lys Ser Asn Leu Asp
                240                 245                 250

GAA GAG GTG AAT GTA ATT CCT CCA CAC ACT CCA GTT AGG ACT GTT ATG         819
Glu Glu Val Asn Val Ile Pro Pro His Thr Pro Val Arg Thr Val Met
            255                 260                 265

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Asn Thr Ile Gln Gln Leu Met Met Ile Leu Asn Ser Ala Ser Asp Gln
        270                 275                 280
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CCT TCA GAA AAT CTG ATT TCC TAT TTT AAC AAC TGC ACA GTG AAT CCA      915
Pro Ser Glu Asn Leu Ile Ser Tyr Phe Asn Asn Cys Thr Val Asn Pro
    285                 290                 295

AAA GAA AGT ATA CTG AAA AGA GTG AAG GAT ATA GGA TAC ATC TTT AAA      963
Lys Glu Ser Ile Leu Lys Arg Val Lys Asp Ile Gly Tyr Ile Phe Lys
300                 305                 310                 315

GAG AAA TTT GCT AAA GCT GTG GGA CAG GGT TGT GTC GAA ATT GGA TCA     1011
Glu Lys Phe Ala Lys Ala Val Gly Gln Gly Cys Val Glu Ile Gly Ser
                320                 325                 330

CAG CGA TAC AAA CTT GGA GTT CGC TTG TAT TAC CGA GTA ATG GAA TCC     1059
Gln Arg Tyr Lys Leu Gly Val Arg Leu Tyr Tyr Arg Val Met Glu Ser
            335                 340                 345

ATG CTT AAA TCA GAA GAA GAA CGA TTA TCC ATT CAA AAT TTT AGC AAA     1107
Met Leu Lys Ser Glu Glu Glu Arg Leu Ser Ile Gln Asn Phe Ser Lys
        350                 355                 360

CTT CTG AAT GAC AAC ATT TTT CAT ATG TCT TTA TTG GCG TGC GCT CTT     1155
Leu Leu Asn Asp Asn Ile Phe His Met Ser Leu Leu Ala Cys Ala Leu
    365                 370                 375

GAG GTT GTA ATG GCC ACA TAT AGC AGA AGT ACA TCT CAG AAT CTT GAT     1203
Glu Val Val Met Ala Thr Tyr Ser Arg Ser Thr Ser Gln Asn Leu Asp
380                 385                 390                 395

TCT GGA ACA GAT TTG TCT TTC CCA TGG ATT CTG AAT GTG CTT AAT TTA     1251
Ser Gly Thr Asp Leu Ser Phe Pro Trp Ile Leu Asn Val Leu Asn Leu
                400                 405                 410

AAA GCC TTT GAT TTT TAC AAA GTG ATC GAA AGT TTT ATC AAA GCA GAA     1299
Lys Ala Phe Asp Phe Tyr Lys Val Ile Glu Ser Phe Ile Lys Ala Glu
            415                 420                 425

GGC AAC TTG ACA AGA GAA ATG ATA AAA CAT TTA GAA CGA TGT GAA CAT     1347
Gly Asn Leu Thr Arg Glu Met Ile Lys His Leu Glu Arg Cys Glu His
        430                 435                 440

CGA ATC ATG GAA TCC CTT GCA TGG CTC TCA GAT TCA CCT TTA TTT GAT     1395
Arg Ile Met Glu Ser Leu Ala Trp Leu Ser Asp Ser Pro Leu Phe Asp
    445                 450                 455

CTT ATT AAA CAA TCA AAG GAC CGA GAA GGA CCA ACT GAT CAC CTT GAA     1443
Leu Ile Lys Gln Ser Lys Asp Arg Glu Gly Pro Thr Asp His Leu Glu
460                 465                 470                 475

TCT GCT TGT CCT CTT AAT CTT CCT CTC CAG AAT AAT CAC ACT GCA GCA     1491
Ser Ala Cys Pro Leu Asn Leu Pro Leu Gln Asn Asn His Thr Ala Ala
                480                 485                 490

GAT ATG TAT CTT TCT CCT GTA AGA TCT CCA AAG AAA AAA GGT TCA ACT     1539
Asp Met Tyr Leu Ser Pro Val Arg Ser Pro Lys Lys Lys Gly Ser Thr
            495                 500                 505

ACG CGT GTA AAT TCT ACT GCA AAT GCA GAG ACA CAA GCA ACC TCA GCC     1587
Thr Arg Val Asn Ser Thr Ala Asn Ala Glu Thr Gln Ala Thr Ser Ala
        510                 515                 520

TTC CAG ACC CAG AAG CCA TTG AAA TCT ACC TCT CTT TCA CTG TTT TAT     1635
Phe Gln Thr Gln Lys Pro Leu Lys Ser Thr Ser Leu Ser Leu Phe Tyr
    525                 530                 535

AAA AAA GTG TAT CGG CTA GCC TAT CTC CGG CTA AAT ACA CTT TGT GAA     1683
Lys Lys Val Tyr Arg Leu Ala Tyr Leu Arg Leu Asn Thr Leu Cys Glu
540                 545                 550                 555
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CGC CTT CTG TCT GAG CAC CCA GAA TTA GAA CAT ATC ATC TGG ACC CTT      1731
Arg Leu Leu Ser Glu His Pro Glu Leu Glu His Ile Ile Trp Thr Leu
                560                 565                 570

TTC CAG CAC ACC CTG CAG AAT GAG TAT GAA CTC ATG AGA GAC AGG CAT      1779
Ph  Gln His Thr Leu Gln Asn Glu Tyr Glu Leu Met Arg Asp Arg His
            575                 580                 585

TTG GAC CAA ATT ATG ATG TGT TCC ATG TAT GGC ATA TGC AAA GTG AAG      1827
Leu Asp Gln Ile Met Met Cys Ser Met Tyr Gly Ile Cys Lys Val Lys
        590                 595                 600

AAT ATA GAC CTT AAA TTC AAA ATC ATT GTA ACA GCA TAC AAG GAT CTT      1875
Asn Ile Asp Leu Lys Phe Lys Ile Ile Val Thr Ala Tyr Lys Asp Leu
    605                 610                 615

CCT CAT GCT GTT CAG GAG ACA TTC AAA CGT GTT TTG ATC AAA GAA GAG      1923
Pro His Ala Val Gln Glu Thr Phe Lys Arg Val Leu Ile Lys Glu Glu
620                 625                 630                 635

GAG TAT GAT TCT ATT ATA GTA TTC TAT AAC TCG GTC TTC ATG CAG AGA      1971
Glu Tyr Asp Ser Ile Ile Val Phe Tyr Asn Ser Val Phe Met Gln Arg
                640                 645                 650

CTG AAA ACA AAT ATT TTG CAG TAT GCT TCC ACC AGG CCC CCT ACC TTG      2019
Leu Lys Thr Asn Ile Leu Gln Tyr Ala Ser Thr Arg Pro Pro Thr Leu
            655                 660                 665

TCA CCA ATA CCT CAC ATT CCT CGA AGC CCT TAC AAG TTT CCT AGT TCA      2067
Ser Pro Ile Pro His Ile Pro Arg Ser Pro Tyr Lys Phe Pro Ser Ser
        670                 675                 680

CCC TTA CGG ATT CCT GGA GGG AAC ATC TAT ATT TCA CCC CTG AAG AGT      2115
Pro Leu Arg Ile Pro Gly Gly Asn Ile Tyr Ile Ser Pro Leu Lys Ser
    685                 690                 695

CCA TAT AAA ATT TCA GAA GGT CTG CCA ACA CCA ACA AAA ATG ACT CCA      2163
Pro Tyr Lys Ile Ser Glu Gly Leu Pro Thr Pro Thr Lys Met Thr Pro
700                 705                 710                 715

AGA TCA AGA ATC TTA GTA TCA ATT GGT GAA TCA TTC GGG ACT TCT GAG      2211
Arg Ser Arg Ile Leu Val Ser Ile Gly Glu Ser Phe Gly Thr Ser Glu
                720                 725                 730

AAG TTC CAG AAA ATA AAT CAG ATG GTA TGT AAC AGC GAC CGT GTG CTC      2259
Lys Phe Gln Lys Ile Asn Gln Met Val Cys Asn Ser Asp Arg Val Leu
            735                 740                 745

AAA AGA AGT GCT GAA GGA AGC AAC CCT CCT AAA CCA CTG AAA AAA CTA      2307
Lys Arg Ser Ala Glu Gly Ser Asn Pro Pro Lys Pro Leu Lys Lys Leu
        750                 755                 760

CGC TTT GAT ATT GAA GGA TCA GAT GAA GCA GAT GGA AGT AAA CAT CTC      2355
Arg Phe Asp Ile Glu Gly Ser Asp Glu Ala Asp Gly Ser Lys His Leu
    765                 770                 775

CCA GGA GAG TCC AAA TTT CAG CAG AAA CTG GCA GAA ATG ACT TCT ACT      2403
Pro Gly Glu Ser Lys Phe Gln Gln Lys Leu Ala Glu Met Thr Ser Thr
780                 785                 790                 795

CGA ACA CGA ATG CAA AAG CAG AAA ATG AAT GAT AGC ATG GAT ACC TCA      2451
Arg Thr Arg Met Gln Lys Gln Lys Met Asn Asp Ser Met Asp Thr Ser
                800                 805                 810

AAC AAG GAA GAG AAA TGAGGATCTC AGGACCTTGG TGGACACTGT GTACACCTCT      2506
Asn Lys Glu Glu Lys
            815
```

GGATTCATTG TCTCTCACAG ATGTGACTGT ATAACTTTCC CAGGTTCTGT TTATGGCCAC 2566

ATTTAATATC TTCAGCTCTT TTTGTGGATA TAAAATGTGC AGATGCAATT GTTTGGGTGA 2626

TTCCTAAGCC ACTTGAAATG TTAGTCATTG TTATTTATAC AAGATTGAAA ATCTTGTGTA 2686

AATCCTGCCA TTTAAAAAGT TGTAGCAGAT TGTTTCCTCT TCCAAAGTAA AATTGCTGTG 2746

CTTTATGGAT AGTAAGAATG GCCCTAGAGT GGGAGTCCTG ATAACCCAGG CCTGTCTGAC 2806

TACTTTGCCT TCTTTTGTAG CATATAGGTG ATGTTTGCTC TTGTTTTTAT TAATTTATAT 2866

GTATATTTTT TTAATTTAAC ATGAACACCC TTAGAAAATG TGTCCTATCT ATCATCCAAA 2926

TGCAATTTGA TTGACTGCCC ATTCACCAAA ATTATCCTGA ACTCTTCTGC AAAAATGGAT 2986

ATTATTAGAA ATTAGAAAAA AATTACTAAT TTTACACATT AGATTTTATT TTACTATTGG 3046

AATCTGATAT ACTGTGTGCT TGTTTTATAA AATTTTGCTT TTAATTAAAT AAAAGCTGGA 3106

AGCAAAGTAT AACCATATGA TACTATCATA CTACTGAAAC AGATTTCATA CCTCAGAATG 3166

TAAAAGAACT TACTGATTAT TTTCTTCATC CAACTTATGT TTTTAAATGA GGATTATTGA 3226

TAGTGG 3232

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3232 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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ATATCAGATT CCAATAGTAA AATAAAATCT AATGTGTAAA ATTAGTAATT TTTTTCTAAT 240

TTCTAATAAT ATCCATTTTT GCAGAAGAGT TCAGGATAAT TTTGGTGAAT GGGCAGTCAA 300

TCAAATTGCA TTTGGATGAT AGATAGGACA CATTTTCTAA GGGTGTTCAT GTTAAATTAA 360

AAAAATATAC ATATAAATTA ATAAAAACAA GAGCAAACAT CACCTATATG CTACAAAAGA 420

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GGCTTAGGAA TCACCCAAAC AATTGCATCT GCACATTTTA TATCCACAAA AAGAGCTGAA 660

GATATTAAAT GTGGCCATAA ACAGAACCTG GGAAAGTTAT ACAGTCACAT CTGTGAGAGA 720

CAATGAATCC AGAGGTGTAC ACAGTGTCCA CCAAGGTCCT GAGATCCTCA TTTCTCTTCC 780

TTGTTTGAGG TATCCATGCT ATCATTCATT TTCTGCTTTT GCATTCGTGT TCGAGTAGAA 840

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TAAATTTCTT CGTATCGTTT AGAAAGATTT TCAACCTCTG GAAGTCCATT AGATGTTACA 2640

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ATATTACATT CATGTTCTTT ACAGAGAACT TCAATAATTC TTGTATCATT TTCTAGTTGT 2760

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ATTTCAGTAG ATATCGAACT GCTGGGTTGT GTCAAATATA TAAGTTCACA TGTCCTTTCC 3060

AATTTGCTGA AGAGTGCAAA CAATACATCA TACTTCTTCA ACAGTCTTGA CATAGCATTA 3120

TCAACTTTGG TACTGGTATC AATTTCTTTT AGTAAGTTAA AGAATTTATG GACACTGATT 3180

TCTATGTTTT TCTGTAGCTC AGTAAAAGTG AACGACATCT CATCTAGGTC GG 3232

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 816 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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 1               5                  10                  15

His Lys Phe Phe Asn Leu Leu Lys Glu Ile Asp Thr Ser Thr Lys Val
            20                  25                  30

Asp Asn Ala Met Ser Arg Leu Leu Lys Lys Tyr Asp Val Leu Phe Ala
        35                  40                  45

Leu Phe Ser Lys Leu Glu Arg Thr Cys Glu Leu Ile Tyr Leu Thr Gln
    50                  55                  60

Pro Ser Ser Ser Ile Ser Thr Glu Ile Asn Ser Ala Leu Val Leu Lys
65                  70                  75                  80

Val Ser Trp Ile Thr Phe Leu Leu Ala Lys Gly Glu Val Leu Gln Met
                85                  90                  95

Glu Asp Asp Leu Val Ile Ser Phe Gln Leu Met Leu Cys Val Leu Asp
            100                 105                 110

Tyr Phe Ile Lys Leu Ser Pro Pro Met Leu Leu Lys Glu Pro Tyr Lys
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Thr Ala Val Ile Pro Ile Asn Gly Ser Pro Arg Thr Pro Arg Arg Gly
    130                 135                 140

Gln Asn Arg Ser Ala Arg Ile Ala Lys Gln Leu Glu Asn Asp Thr Arg
145                 150                 155                 160

Ile Ile Glu Val Leu Cys Lys Glu His Glu Cys Asn Ile Asp Glu Val
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Lys Asn Val Tyr Phe Lys Asn Phe Ile Pro Phe Met Asn Ser Leu Gly
            180                 185                 190

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Tyr Glu Glu Ile Tyr Leu Lys Asn Lys Asp Leu Asp Ala Arg Leu Phe
    210                 215                 220
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Leu Asp His Asp Lys Thr Leu Gln Thr Asp Ser Ile Asp Ser Phe Glu
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Thr Gln Arg Thr Pro Arg Lys Ser Asn Leu Asp Glu Glu Val Asn Val
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260 265 270

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275 280 285

Ile Ser Tyr Phe Asn Asn Cys Thr Val Asn Pro Lys Glu Ser Ile Leu
290 295 300

Lys Arg Val Lys Asp Ile Gly Tyr Ile Phe Lys Glu Lys Phe Ala Lys
305 310 315 320

Ala Val Gly Gln Gly Cys Val Glu Ile Gly Ser Gln Arg Tyr Lys Leu
325 330 335

Gly Val Arg Leu Tyr Tyr Arg Val Met Glu Ser Met Leu Lys Ser Glu
340 345 350

Glu Glu Arg Leu Ser Ile Gln Asn Phe Ser Lys Leu Leu Asn Asp Asn
355 360 365

Ile Phe His Met Ser Leu Leu Ala Cys Ala Leu Glu Val Val Met Ala
370 375 380

Thr Tyr Ser Arg Ser Thr Ser Gln Asn Leu Asp Ser Gly Thr Asp Leu
385 390 395 400

Ser Phe Pro Trp Ile Leu Asn Val Leu Asn Leu Lys Ala Phe Asp Phe
405 410 415

Tyr Lys Val Ile Glu Ser Phe Ile Lys Ala Glu Gly Asn Leu Thr Arg
420 425 430

Glu Met Ile Lys His Leu Glu Arg Cys Glu His Arg Ile Met Glu Ser
435 440 445

Leu Ala Trp Leu Ser Asp Ser Pro Leu Phe Asp Leu Ile Lys Gln Ser
450 455 460

Lys Asp Arg Glu Gly Pro Thr Asp His Leu Glu Ser Ala Cys Pro Leu
465 470 475 480

Asn Leu Pro Leu Gln Asn Asn His Thr Ala Ala Asp Met Tyr Leu Ser
485 490 495

Pro Val Arg Ser Pro Lys Lys Lys Gly Ser Thr Thr Arg Val Asn Ser
500 505 510

Thr Ala Asn Ala Glu Thr Gln Ala Thr Ser Ala Phe Gln Thr Gln Lys
515 520 525

Pro Leu Lys Ser Thr Ser Leu Ser Leu Phe Tyr Lys Lys Val Tyr Arg
530 535 540

Leu Ala Tyr Leu Arg Leu Asn Thr Leu Cys Glu Arg Leu Leu Ser Glu
545 550 555 560

His Pro Glu Leu Glu His Ile Ile Trp Thr Leu Phe Gln His Thr Leu
565 570 575

Gln Asn Glu Tyr Glu Leu Met Arg Asp Arg His Leu Asp Gln Ile Met

580 585 590

Met Cys Ser Met Tyr Gly Ile Cys Lys Val Lys Asn Ile Asp Leu Lys
595 600 605

Phe Lys Ile Ile Val Thr Ala Tyr Lys Asp Leu Pro His Ala Val Gln
610 615 620

Glu Thr Phe Lys Arg Val Leu Ile Lys Glu Glu Glu Tyr Asp Ser Ile
625 630 635 640

Ile Val Phe Tyr Asn Ser Val Phe Met Gln Arg Leu Lys Thr Asn Ile
645 650 655

Leu Gln Tyr Ala Ser Thr Arg Pro Pro Thr Leu Ser Pro Ile Pro His
660 665 670

Ile Pro Arg Ser Pro Tyr Lys Phe Pro Ser Ser Pro Leu Arg Ile Pro
675 680 685

Gly Gly Asn Ile Tyr Ile Ser Pro Leu Lys Ser Pro Tyr Lys Ile Ser
690 695 700

Glu Gly Leu Pro Thr Pro Thr Lys Met Thr Pro Arg Ser Arg Ile Leu
705 710 715 720

Val Ser Ile Gly Glu Ser Phe Gly Thr Ser Glu Lys Phe Gln Lys Ile
725 730 735

Asn Gln Met Val Cys Asn Ser Asp Arg Val Leu Lys Arg Ser Ala Glu
740 745 750

Gly Ser Asn Pro Pro Lys Pro Leu Lys Lys Leu Arg Phe Asp Ile Glu
755 760 765

Gly Ser Asp Glu Ala Asp Gly Ser Lys His Leu Pro Gly Glu Ser Lys
770 775 780

Phe Gln Gln Lys Leu Ala Glu Met Thr Ser Thr Arg Thr Arg Met Gln
785 790 795 800

Lys Gln Lys Met Asn Asp Ser Met Asp Thr Ser Asn Lys Glu Glu Lys
805 810 815

International Application No: PCT/

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 67, lines 25-37 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet

Name of depositary institution

American Type Culture Collection

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, MD 20852
US

Date of deposit February 10, 1993 Accession Number 69240

B. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on a separate attached sheet

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☑ This sheet was received with the International application when filed (to be checked by the receiving Office)

C. Williams
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

Form PCT/RO/134 (cont.)

American Type Culture Collection

12301 Parklawn Drive
Rockville, MD 20852
US

Accession No.	Date of Deposit
69241	February 10, 1993

We claim:

1. A method of treating a disease characterized by abnormal cellular proliferation in a mammal by a process comprising the steps of:

a. administering an effective dose of a $p94^{RB}$ encoding expression vector to a mammal having a disease characterized by abnormally proliferating cells, such that said expression vector is inserted into said abnormally proliferating cells, said expression vector comprising a gene encoding $p94^{RB}$; and

b. expressing $p94^{RB}$ in said abnormally proliferating cells in an amount effective to suppress proliferation of said abnormally proliferating cells; and wherein said $p94^{RB}$ encoding expression vector comprises a $p94^{RB}$ encoding gene, said gene encoding a protein having an amino acid sequence substantially according to SEQ ID NO:3, provided that said protein is not $p110^{RB}$.

2. The method according to claim 1 wherein said $p94^{RB}$ encoding gene encodes a protein having an amino acid sequence according to SEQ ID NO:3.

3. The method according to claim 1 wherein said $p94^{RB}$ encoding gene has a DNA sequence substantially according to SEQ ID NO:1.

4. The method according to claim 3 wherein said $p94^{RB}$ encoding gene has a DNA sequence according to SEQ ID NO:1.

5. The method according to claim 2 wherein said expression vector is selected from the group consisting of a plasmid and a viral vector.

6. The method according to claim 4 wherein said expression vector is selected from the group consisting of a plasmid and a viral vector.

7. The method according to claim 5 wherein said viral vector is selected from a group consisting of a retroviral vector, an adenoviral vector and a herpesviral vector, wherein said $p94^{RB}$ encoding gene is under the control of a promoter selected from the group consisting of a retroviral promoter, an adenoviral promoter, a CMV promoter and a ß-actin promoter.

8. The method according to claim 6 wherein said viral vector is selected from a group consisting of a retroviral vector, an adenoviral vector and a herpesviral vector wherein said $p94^{RB}$ encoding gene is under the control of a promoter selected from the group consisting of a retroviral promoter, an adenoviral promoter, a CMV promoter and a ß-actin promoter.

9. The method according to claim 8 wherein said expression vector is selected from the group consisting of plasmid pCMV-s-RB42 and plasmid pßA-s-RB34.

10. The method according to claims 7, 8 or 9 wherein said expression vector is inserted into said abnormally proliferating cells by a method selected from the group consisting of viral infection or transduction, liposome-mediated transfection, polybrene-mediated transfection and $CaPO_4$ mediated transfection.

11. The method according to claim 1 wherein said abnormally proliferating cells are tumor or cancer cells and said mammal is a human.

12. The method according to claim 11 wherein said tumor or cancer cells are selected from the group consisting of carcinoma and sarcoma cells.

13. The method according to claim 11 wherein tumor or cancer cells are selected from the group consisting of a bladder carcinoma, a lung carcinoma, a breast carcinoma, a prostate carcinoma, a fibrosarcoma, an osteosarcoma, and a cervical carcinoma.

14. The method according to claim 11 wherein said tumor or cancer cells are cells having at least one genetically defective tumor suppressor gene or oncogene selected from the group consisting of an RB, a p53, a c-*myc* an N-*ras* and a c-*yes*-1 gene.

15. The method according to claim 11 wherein said tumor or cancer cells have no detectable genetic defect of a tumor suppressor gene, and the tumor suppressor gene is selected from the group consisting of an RB gene and a p53 gene.

16. The method according to claim 12 wherein said carcinoma cells are bladder carcinoma cells and said step of administering said expression vector to treat said bladder carcinoma cells is by means of an infusion of said expression vector into a bladder in need of such treatment.

17. A DNA molecule encoding $p94^{RB}$ having an amino acid sequence substantially according to SEQ ID NO:3,

provided that said DNA molecule does not also code for $p110^{RB}$.

18. The DNA molecule according to claim 17, said DNA molecule coding for a protein having an amino acid sequence according to SEQ ID NO:3.

19. The DNA molecule according to claim 17, said DNA molecule having a DNA sequence substantially according to SEQ ID NO:1, provided that said DNA molecule does not also code for $p110^{RB}$.

20. The DNA molecule according to claim 17, said DNA molecule having a DNA sequence according to SEQ ID NO:1.

21. An expression vector comprising said DNA molecule according to claim 18, capable of inserting said $p94^{RB}$ encoding DNA molecule into a mammalian host cell and of expressing $p94^{RB}$ therein.

22. An expression vector comprising said DNA molecule according to claim 20, capable of inserting said $p94^{RB}$ encoding DNA molecule into a mammalian host cell and of expressing $p94^{RB}$ therein.

23. The expression vector according to claim 21, wherein said expression vector is selected from the group consisting of a plasmid and a viral vector.

24. The expression vector according to claim 22, wherein said expression vector is selected from the group consisting of a plasmid and a viral vector.

25. The expression vector according to claim 23 wherein said viral vector is selected from a group consisting of a retroviral vector, an adenoviral

vector and a herpesviral vector, and wherein said $p94^{RB}$ encoding gene is under the control of a promoter selected from the group consisting of a retroviral promoter, an adenoviral promoter, a CMV promoter and a ß-actin promoter.

26. The expression vector according to claim 24 wherein said viral vector is selected from a group consisting of a retroviral vector, an adenoviral vector and a herpesviral vector and wherein said $p94^{RB}$ encoding gene is under the control of a promoter selected from the group consisting of a retroviral promoter, an adenoviral promoter, a CMV promoter and a ß-actin promoter.

27. The expression vector according to claim 24 wherein said expression vector is plasmid pCMV-s-RB42 and plasmid pßA-s-RB34.

28. A composition suitable for treating a tumor or cancer in a mammal comprising an effective amount of the expression vector according to claim 21, together with a suitable carrier or vehicle.

29. A composition suitable for treating a tumor or cancer in a mammal comprising an effective amount of the expression vector according to claim 22, together with a suitable carrier or vehicle.

30. A composition according to claims 28 or 29 wherein said carrier or vehicle comprises an encapsulating liposome.

31. A $p94^{RB}$ protein comprising a polypeptide having an amino acid sequence substantially according to SEQ ID NO:3; provided that said protein is not $p110^{RB}$.

32. A $p94^{RB}$ protein according to claim 31 comprising a polypeptide having an amino acid sequence according to SEQ ID NO:3.

33. A composition suitable for treating a tumor or cancer in a mammal comprising an effective amount of $p94^{RB}$ according to claim 31, together with a suitable carrier or vehicle.

34. A composition suitable for treating a tumor or cancer in a mammal comprising an effective amount of $p94^{RB}$ according to claim 32, together with a suitable carrier or vehicle.

35. A composition according to claims 33 or 34 wherein said carrier or vehicle comprises an encapsulating liposome.

36. A method of producing a $p94^{RB}$ protein comprising the steps of:

a. inserting a compatible expression vector comprising a $p94^{RB}$ encoding gene into a host cell; and

b. causing said host cell to express $p94^{RB}$ protein.

37. The method according to claim 36 wherein said host cell is selected from the group consisting of a prokaryotic host cell and a eukaryotic host cell.

38. The method according to claim 32 wherein said eukaryotic host cell is a mammalian host cell and said expression vector is compatible with said mammalian host cell.

39. The method according to claim 38 wherein said expression vector is selected from the group

consisting of plasmid pCMV-s-RB42 and plasmid pβA-s-RB34.

40. The method according to claim 37 wherein said host cell is an insect host cell and said expression vector is a plasmid or a viral vector compatible with said insect host cell.

41. The method according to claim 40 wherein said baculovirus vector is AcMNPV-RB94.

42. A method of treating abnormally proliferating cells of a mammal *ex vivo* by a process comprising the steps of:

a. removing a tissue sample in need of treatment from a mammal, said tissue sample comprising abnormally proliferating cells;

b. contacting said tissue sample in need of treatment with an effective dose of a $p94^{RB}$ encoding expression vector;

c. expressing said $p94^{RB}$ in said abnormally proliferating cells in amounts effective to suppress proliferation of said abnormally proliferating cells; and

d. returning said treated tissue sample to said mammal or placing said tissue sample into another mammal.

43. A method of treating a disease characterized by abnormal cellular proliferation in a mammal by administering $p94^{RB}$ protein to a mammal having a disease characterized by abnormally proliferating cells, such that said $p94^{RB}$ protein is inserted into said abnormally proliferating cells in amounts effective to suppress abnormal proliferation of said cells.

44. The method according to claim 43 wherein said $p94^{RB}$ protein has an amino acid sequence substantially according to SEQ ID NO:3, provided that said protein is not $p110^{RB}$.

45. The method according to claim 43 wherein said $p94^{RB}$ protein has an amino acid sequence according to SEQ ID NO:3.

46. The method according to claim 43 wherein said abnormally proliferating cells are tumor or cancer cells, and said mammal is a human patient.

47. The method according to claim 46 wherein said $p94^{RB}$ protein is encapsulated in a liposome carrier and said $p94^{RB}$ protein is inserted into said abnormally proliferating cells by fusion of said liposome encapsulated $p94^{RB}$ protein with said abnormally proliferating cells.

48. The method according to claim 46 wherein said tumor or cancer cells are selected from the group consisting of a bladder carcinoma, a lung carcinoma, a breast carcinoma, a prostate carcinoma, a fibrosarcoma, an osteosarcoma, and a cervix carcinoma.

49. The method according to claim 46 wherein said tumor or cancer cells are cells having one or more genetically defective tumor suppressor genes and oncogenes selected from the group consisting of an RB, a p53, a c-myc, an N-ras and a c-yes-1 gene.

50. The method according to claim 46 wherein said tumor or cancer cells are cells having no detectable genetic defect of a tumor suppressor gene selected from the group consisting of an RB gene and a p53 gene.

51. The method according to claim 48 wherein said tumor or cancer cells are lung carcinoma cells and said step of administering said $p94^{RB}$ protein to treat said lung carcinoma cells is by means of an infusion of said liposome-encapsulated $p94^{RB}$ protein into the respiratory tract and the pulmonary area in need of such treatment.

52. A method of treating abnormally proliferating cells of a mammal *ex vivo* by a process comprising the steps of:

a. removing a tissue sample in need of treatment from a mammal, said tissue sample comprising abnormally proliferating cells;

b. contacting said tissue sample in need of treatment with an effective dose of a $p94^{RB}$ protein; and

c. returning said treated tissue sample to said mammal or placing said tissue sample into another mammal; and said $p94^{RB}$ protein has the amino acid sequence substantially according to SEQ ID NO:3.

53. A method of treating abnormally proliferating cells by a process comprising the steps of:

a. inserting a $p94^{RB}$ encoding expression vector into abnormally proliferating cells of a mammal; and

b. expressing said $p94^{RB}$ therein in amounts effective to suppress proliferation of said abnormally proliferating cells and said $p94^{RB}$ encoding expression vector comprises a $p94^{RB}$ encoding gene, said gene encoding a protein having an amino acid sequence substantially according to SEQ ID NO:3.

AMENDED CLAIMS

[received by the International Bureau on 11 August 1994 (11.08.94); original claim 38 amended; remaining claims unchanged (1 page)]

32. A $p94^{RB}$ protein according to claim 31 comprising a polypeptide having an amino acid sequence according to SEQ ID NO:3.

33. A composition suitable for treating a tumor or cancer in a mammal comprising an effective amount of $p94^{RB}$ according to claim 31, together with a suitable carrier or vehicle.

34. A composition suitable for treating a tumor or cancer in a mammal comprising an effective amount of $p94^{RB}$ according to claim 32, together with a suitable carrier or vehicle.

35. A composition according to claims 33 or 34 wherein said carrier or vehicle comprises an encapsulating liposome.

36. A method of producing a $p94^{RB}$ protein comprising the steps of:

a. inserting a compatible expression vector comprising a $p94^{RB}$ encoding gene into a host cell; and

b. causing said host cell to express $p94^{RB}$ protein.

37. The method according to claim 36 wherein said host cell is selected from the group consisting of a prokaryotic host cell and a eukaryotic host cell.

38. The method according to claim 37 wherein said eukaryotic host cell is a mammalian host cell and said expression vector is compatible with said mammalian host cell.

39. The method according to claim 38 wherein said expression vector is selected from the group

```
                10                  20                  30                  40                  50                  60
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3'      GGCTGG ATCTACTCTA CAGCAAGTGA AAATGACTCG ATGTCTTTTT GTATCTTTAG
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   TCACAGGTAT TTAAGAAATT GAATGATTTT CTTTAACTAT GGTCATGGTT TCAACTATTA
               130                 140                 150                 160                 170                 180
   GCTATGTCAA GACTGTTGAA GAAGTATGAT GTATTGTTTG CACTCTTCAG CAAATTGGAA
   CGATACAGTT CTGACAACTT CTTCATACTA CATAACAAAC GTGAGAAGTC GTTTAACCTT
               190                 200                 210                 220                 230                 240
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   TCCTGTACAC TTGAATATAT AAACTGTGTT GGGTCGTCAA GCTATAGATG ACTTTATTTA
               250                 260                 270                 280                 290                 300
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               310                 320                 330                 340                 350                 360
   CAAATGGAAG ATGATCTGGT GATTTCATTT CAGTTAATGC TATGTGTCCT TGACTATTTT
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   TTACCAAGTG GAGCTTGTGG GTCCGCTCCA GTCTTGTCCT CACGTGCCTA TCGTTTTGTT
               490                 500                 510                 520                 530                 540
   CTAGAAAATG ATACAAGAAT TATTGAAGTT CTCTGTAAAG AACATGAATG TAATATAGAT
   GATCTTTTAC TATGTTCTTA ATAACTTCAA GAGACATTTC TTGTACTTAC ATTATATCTA
```

FIG. 1A

PCT/US94/03211

```
       550         560         570         580         590         600
GAGGTGAAAA ATGTTTATTT CAAAAATTTT ATACCTTTTA TGAATTCTCT TGGACTTGTA
CTCCACTTTT TACAAATAAA GTTTTTAAAA TATGGAAAAT ACTTAAGAGA ACCTGAACAT
       610         620         630         640         650         660
ACATCTAATG GACTTCCAGA GGTTGAAAAT CTTTCTAAAC GATACGAAGA AATTTATCTT
TGTAGATTAC CTGAAGGTCT CCAACTTTTA GAAAGATTTG CTATGCTTCT TTAAATAGAA
       670         680         690         700         710         720
AAAAATAAAG ATCTAGATGC AAGATTATTT TTGGATCATG ATAAAACTCT TCAGACTGAT
TTTTTATTTC TAGATCTACG TTCTAATAAA AACCTAGTAC TATTTTGAGA AGTCTGACTA
       730         740         750         760         770         780
TCTATAGACA GTTTTGAAAC ACAGAGAACA CCACGAAAAA GTAACCTTGA TGAAGAGGTG
AGATATCTGT CAAAACTTTG TGTCTCTTGT GGTGCTTTTT CATTGGAACT ACTTCTCCAC
       790         800         810         820         830         840
AATGTAATTC CTCCACACAC TCCAGTTAGG ACTGTTATGA ACACTATCCA ACAATTAATG
TTACATTAAG GAGGTGTGTG AGGTCAATCC TGACAATACT TGTGATAGGT TGTTAATTAC
       850         860         870         880         890         900
ATGATTTTAA ATTCAGCAAG TGATCAACCT TCAGAAAATC TGATTTCCTA TTTTAACAAC
TACTAAAATT TAAGTCGTTC ACTAGTTGGA AGTCTTTTAG ACTAAAGGAT AAAATTGTTG
       910         920         930         940         950         960
TGCACAGTGA ATCCAAAAGA AAGTATACTG AAAAGAGTGA AGGATATAGG ATACATCTTT
ACGTGTCACT TAGGTTTTCT TTCATATGAC TTTTCTCACT TCCTATATCC TATGTAGAAA
       970         980         990        1000        1010        1020
AAAGAGAAAT TTGCTAAAGC TGTGGGACAG GGTTGTGTCG AAATTGGATC ACAGCGATAC
TTTCTCTTTA AACGATTTCG ACACCCTGTC CCAACACAGC TTTAACCTAG TGTCGCTATG
      1030        1040        1050        1060        1070        1080
AAACTTGGAG TTCGCTTGTA TTACCGAGTA ATGGAATCCA TGCTTAAATC AGAAGAAGAA
TTTGAACCTC AAGCGAACAT AATGGCTCAT TACCTTAGGT ACGAATTTAG TCTTCTTCTT
```

FIG. 1B

```
      1090        1100        1110        1120        1130        1140
CGATTATCCA TTCAAAATTT TAGCAAACTT CTGAATGACA ACATTTTTCA TATGTCTTTA
GCTAATAGGT AAGTTTTAAA ATCGTTTGAA GACTTACTGT TGTAAAAAGT ATACAGAAAT
      1150        1160        1170        1180        1190        1200
TTGGCGTGCG CTCTTGAGGT TGTAATGGCC ACATATAGCA GAAGTACATC TCAGAATCTT
AACCGCACGC GAGAACTCCA ACATTACCGG TGTATATCGT CTTCATGTAG AGTCTTAGAA
      1210        1220        1230        1240        1250        1260
GATTCTGGAA CAGATTTGTC TTTCCCATGG ATTCTGAATG TGCTTAATTT AAAAGCCTTT
CTAAGACCTT GTCTAAACAG AAAGGGTACC TAAGACTTAC ACGAATTAAA TTTTCGGAAA
      1270        1280        1290        1300        1310        1320
GATTTTTACA AAGTGATCGA AAGTTTTATC AAAGCAGAAG GCAACTTGAC AAGAGAAATG
CTAAAAATGT TTCACTAGCT TTCAAAATAG TTTCGTCTTC CGTTGAACTG TTCTCTTTAC
      1330        1340        1350        1360        1370        1380
ATAAAACATT TAGAACGATG TGAACATCGA ATCATGGAAT CCCTTGCATG GCTCTCAGAT
TATTTTGTAA ATCTTGCTAC ACTTGTAGCT TAGTACCTTA GGGAACGTAC CGAGAGTCTA
      1390        1400        1410        1420        1430        1440
TCACCTTTAT TTGATCTTAT TAAACAATCA AAGGACCGAG AAGGACCAAC TGATCACCTT
AGTGGAAATA AACTAGAATA ATTTGTTAGT TTCCTGGCTC TTCCTGGTTG ACTAGTGGAA
      1450        1460        1470        1480        1490        1500
GAATCTGCTT GTCCTCTTAA TCTTCCTCTC CAGAATAATC ACACTGCAGC AGATATGTAT
CTTAGACGAA CAGGAGAATT AGAAGGAGAG GTCTTATTAG TGTGACGTCG TCTATACATA
      1510        1520        1530        1540        1550        1560
CTTTCTCCTG TAAGATCTCC AAAGAAAAAA GGTTCAACTA CGCGTGTAAA TTCTACTGCA
GAAAGAGGAC ATTCTAGAGG TTTCTTTTTT CCAAGTTGAT GCGCACATTT AAGATGACGT
      1570        1580        1590        1600        1610        1620
AATGCAGAGA CACAAGCAAC CTCAGCCTTC CAGACCCAGA AGCCATTGAA ATCTACCTCT
TTACGTCTCT GTGTTCGTTG GAGTCGGAAG GTCTGGGTCT TCGGTAACTT TAGATGGAGA
```

FIG. 1C

```
      1630       1640       1650       1660       1670       1680
CTTTCACTGT TTTATAAAAA AGTGTATCGG CTAGCCTATC TCCGGCTAAA TACACTTTGT
GAAAGTGACA AAATATTTTT TCACATAGCC GATCGGATAG AGGCCGATTT ATGTGAAACA
      1690       1700       1710       1720       1730       1740
GAACGCCTTC TGTCTGAGCA CCCAGAATTA GAACATATCA TCTGGACCCT TTTCCAGCAC
CTTGCGGAAG ACAGACTCGT GGGTCTTAAT CTTGTATAGT AGACCTGGGA AAAGGTCGTG
      1750       1760       1770       1780       1790       1800
ACCCTGCAGA ATGAGTATGA ACTCATGAGA GACAGGCATT TGGACCAAAT TATGATGTGT
TGGGACGTCT TACTCATACT TGAGTACTCT CTGTCCGTAA ACCTGGTTTA ATACTACACA
      1810       1820       1830       1840       1850       1860
TCCATGTATG GCATATGCAA AGTGAAGAAT ATAGACCTTA AATTCAAAAT CATTGTAACA
AGGTACATAC CGTATACGTT TCACTTCTTA TATCTGGAAT TTAAGTTTTA GTAACATTGT
      1870       1880       1890       1900       1910       1920
GCATACAAGG ATCTTCCTCA TGCTGTTCAG GAGACATTCA AACGTGTTTT GATCAAAGAA
CGTATGTTCC TAGAAGGAGT ACGACAAGTC CTCTGTAAGT TTGCACAAAA CTAGTTTCTT
      1930       1940       1950       1960       1970       1980
GAGGAGTATG ATTCTATTAT AGTATTCTAT AACTCGGTCT TCATGCAGAG ACTGAAAACA
CTCCTCATAC TAAGATAATA TCATAAGATA TTGAGCCAGA AGTACGTCTC TGACTTTTGT
      1990       2000       2010       2020       2030       2040
AATATTTTGC AGTATGCTTC CACCAGGCCC CCTACCTTGT CACCAATACC TCACATTCCT
TTATAAAACG TCATACGAAG GTGGTCCGGG GGATGGAACA GTGGTTATGG AGTGTAAGGA
      2050       2060       2070       2080       2090       2100
CGAAGCCCTT ACAAGTTTCC TAGTTCACCC TTACGGATTC CTGGAGGGAA CATCTATATT
GCTTCGGGAA TGTTCAAAGG ATCAAGTGGG AATGCCTAAG GACCTCCCTT GTAGATATAA
      2110       2120       2130       2140       2150       2160
TCACCCCTGA AGAGTCCATA TAAAATTTCA GAAGGTCTGC CAACACCAAC AAAAATGACT
AGTGGGGACT TCTCAGGTAT ATTTTAAAGT CTTCCAGACG GTTGTGGTTG TTTTTACTGA
```

FIG. 1D

```
      2170        2180        2190        2200        2210        2220
CCAAGATCAA GAATCTTAGT ATCAATTGGT GAATCATTCG GGACTTCTGA GAAGTTCCAG
GGTTCTAGTT CTTAGAATCA TAGTTAACCA CTTAGTAAGC CCTGAAGACT CTTCAAGGTC
      2230        2240        2250        2260        2270        2280
AAAATAAATC AGATGGTATG TAACAGCGAC CGTGTGCTCA AAAGAAGTGC TGAAGGAAGC
TTTTATTTAG TCTACCATAC ATTGTCGCTG GCACACGAGT TTTCTTCACG ACTTCCTTCG
      2290        2300        2310        2320        2330        2340
AACCCTCCTA AACCACTGAA AAAACTACGC TTTGATATTG AAGGATCAGA TGAAGCAGAT
TTGGGAGGAT TTGGTGACTT TTTTGATGCG AAACTATAAC TTCCTAGTCT ACTTCGTCTA
      2350        2360        2370        2380        2390        2400
GGAAGTAAAC ATCTCCCAGG AGAGTCCAAA TTTCAGCAGA AACTGGCAGA AATGACTTCT
CCTTCATTTG TAGAGGGTCC TCTCAGGTTT AAAGTCGTCT TTGACCGTCT TTACTGAAGA
      2410        2420        2430        2440        2450        2460
ACTCGAACAC GAATGCAAAA GCAGAAAATG AATGATAGCA TGGATACCTC AAACAAGGAA
TGAGCTTGTG CTTACGTTTT CGTCTTTTAC TTACTATCGT ACCTATGGAG TTTGTTCCTT
      2470        2480        2490        2500        2510        2520
GAGAAATGAG GATCTCAGGA CCTTGGTGGA CACTGTGTAC ACCTCTGGAT TCATTGTCTC
CTCTTTACTC CTAGAGTCCT GGAACCACCT GTGACACATG TGGAGACCTA AGTAACAGAG
      2530        2540        2550        2560        2570        2580
TCACAGATGT GACTGTATAA CTTTCCCAGG TTCTGTTTAT GGCCACATTT AATATCTTCA
AGTGTCTACA CTGACATATT GAAAGGGTCC AAGACAAATA CCGGTGTAAA TTATAGAAGT
      2590        2600        2610        2620        2630        2640
GCTCTTTTTG TGGATATAAA ATGTGCAGAT GCAATTGTTT GGGTGATTCC TAAGCCACTT
CGAGAAAAAC ACCTATATTT TACACGTCTA CGTTAACAAA CCCACTAAGG ATTCGGTGAA
      2650        2660        2670        2680        2690        2700
GAAATGTTAG TCATTGTTAT TTATACAAGA TTGAAAATCT TGTGTAAATC CTGCCATTTA
CTTTACAATC AGTAACAATA AATATGTTCT AACTTTTAGA ACACATTTAG GACGGTAAAT
```

FIG. 1E

```
      2710        2720        2730        2740        2750        2760
AAAAGTTGTA GCAGATTGTT TCCTCTTCCA AAGTAAAATT GCTGTGCTTT ATGGATAGTA
TTTTCAACAT CGTCTAACAA AGGAGAAGGT TTCATTTTAA CGACACGAAA TACCTATCAT
      2770        2780        2790        2800        2810        2820
AGAATGGCCC TAGAGTGGGA GTCCTGATAA CCCAGGCCTG TCTGACTACT TTGCCTTCTT
TCTTACCGGG ATCTCACCCT CAGGACTATT GGGTCCGGAC AGACTGATGA AACGGAAGAA
      2830        2840        2850        2860        2870        2880
TTGTAGCATA TAGGTGATGT TTGCTCTTGT TTTTATTAAT TTATATGTAT ATTTTTTTAA
AACATCGTAT ATCCACTACA AACGAGAACA AAAATAATTA AATATACATA TAAAAAAATT
      2890        2900        2910        2920        2930        2940
TTTAACATGA ACACCCTTAG AAAATGTGTC CTATCTATCT TCCAAATGCA ATTTGATTGA
AAATTGTACT TGTGGGAATC TTTTACACAG GATAGATAGA AGGTTTACGT TAAACTAACT
      2950        2960        2970        2980        2990        3000
CTGCCCATTC ACCAAAATTA TCCTGAACTC TTCTGCAAAA ATGGATATTA TTAGAAATTA
GACGGGTAAG TGGTTTTAAT AGGACTTGAG AAGACGTTTT TACCTATAAT AATCTTTAAT
      3010        3020        3030        3040        3050        3060
GAAAAAAATT ACTAATTTTA CACATTAGAT TTTATTTTAC TATTGGAATC TGATATACTG
CTTTTTTTAA TGATTAAAAT GTGTAATCTA AAATAAAATG ATAACCTTAG ACTATATGAC
      3070        3080        3090        3100        3110        3120
TGTGCTTGTT TTATAAAATT TTGCTTTTAA TTAAATAAAA GCTGGAAGCA AAGTATAACC
ACACGAACAA AATATTTTAA AACGAAAATT AATTTATTTT CGACCTTCGT TTCATATTGG
      3130        3140        3150        3160        3170        3180
ATATGATACT ATCATACTAC TGAAACAGAT TTCATACCTC AGAATGTAAA AGAACTTACT
TATACTATGA TAGTATGATG ACTTTGTCTA AAGTATGGAG TCTTACATTT TCTTGAATGA
      3190        3200        3210        3220        3230
GATTATTTTC TTCATCCAAC TTATGTTTTT AAATGAGGAT TATTGATAGT GG      3'
CTAATAAAAG AAGTAGGTTG AATACAAAAA TTTACTCCTA ATAACTATCA CCCTAG  5'
```

FIG. 1F

```
     GATCCCGA CCTAGATGAG ATG TCG TTC ACT TTT ACT GAG CTA CAG AAA   48
                         Met Ser Phe Thr Phe Thr Glu Leu Gln Lys   10

AAC ATA GAA ATC AGT GTC CAT AAA TTC TTT AAC TTA CTA AAA GAA ATT   96
Asn Ile Glu Ile Ser Val His Lys Phe Phe Asn Leu Leu Lys Glu Ile   26

GAT ACC AGT ACC AAA GTT GAT AAT GCT ATG TCA AGA CTG TTG AAG AAG  144
Asp Thr Ser Thr Lys Val Asp Asn Ala Met Ser Arg Leu Leu Lys Lys   42

TAT GAT GTA TTG TTT GCA CTC TTC AGC AAA TTG GAA AGG ACA TGT GAA  192
Tyr Asp Val Leu Phe Ala Leu Phe Ser Lys Leu Glu Arg Thr Cys Glu   58

CTT ATA TAT TTG ACA CAA CCC AGC AGT TCG ATA TCT ACT GAA ATA AAT  240
Leu Ile Tyr Leu Thr Gln Pro Ser Ser Ser Ile Ser Thr Glu Ile Asn   74

TCT GCA TTG GTG CTA AAA GTT TCT TGG ATC ACA TTT TTA TTA GCT AAA  288
Ser Ala Leu Val Leu Lys Val Ser Trp Ile Thr Phe Leu Leu Ala Lys   90

GGG GAA GTA TTA CAA ATG GAA GAT GAT CTG GTG ATT TCA TTT CAG TTA  336
Gly Glu Val Leu Gln Met Glu Asp Asp Leu Val Ile Ser Phe Gln Leu  106

ATG CTA TGT GTC CTT GAC TAT TTT ATT AAA CTC TCA CCT CCC ATG TTG  384
Met Leu Cys Val Leu Asp Tyr Phe Ile Lys Leu Ser Pro Pro Met Leu  122

CTC AAA GAA CCA TAT AAA ACA GCT GTT ATA CCC ATT AAT GGT TCA CCT  432
Leu Lys Glu Pro Tyr Lys Thr Ala Val Ile Pro Ile Asn Gly Ser Pro  138

CGA ACA CCC AGG CGA GGT CAG AAC AGG AGT GCA CGG ATA GCA AAA CAA  480
Arg Thr Pro Arg Arg Gly Gln Asn Arg Ser Ala Arg Ile Ala Lys Gln  154
```

FIG. 2A

```
CTA GAA AAT GAT ACA AGA ATT ATT GAA GTT CTC TGT AAA GAA CAT GAA  528
Leu Glu Asn Asp Thr Arg Ile Ile Glu Val Leu Cys Lys Glu His Glu  170

TGT AAT ATA GAT GAG GTG AAA AAT GTT TAT TTC AAA AAT TTT ATA CCT  576
Cys Asn Ile Asp Glu Val Lys Asn Val Tyr Phe Lys Asn Phe Ile Pro  186

TTT ATG AAT TCT CTT GGA CTT GTA ACA TCT AAT GGA CTT CCA GAG GTT  624
Phe Met Asn Ser Leu Gly Leu Val Thr Ser Asn Gly Leu Pro Glu Val  202

GAA AAT CTT TCT AAA CGA TAC GAA GAA ATT TAT CTT AAA AAT AAA GAT  672
Glu Asn Leu Ser Lys Arg Tyr Glu Glu Ile Tyr Leu Lys Asn Lys Asp  218

CTA GAT GCA AGA TTA TTT TTG GAT CAT GAT AAA ACT CTT CAG ACT GAT  720
Leu Asp Ala Arg Leu Phe Leu Asp His Asp Lys Thr Leu Gln Thr Asp  234

TCT ATA GAC AGT TTT GAA ACA CAG AGA ACA CCA CGA AAA AGT AAC CTT  768
Ser Ile Asp Ser Phe Glu Thr Gln Arg Thr Pro Arg Lys Ser Asn Leu  250

GAT GAA GAG GTG AAT GTA ATT CCT CCA CAC ACT CCA GTT AGG ACT GTT  816
Asp Glu Glu Val Asn Val Ile Pro Pro His Thr Pro Val Arg Thr Val  266

ATG AAC ACT ATC CAA CAA TTA ATG ATG ATT TTA AAT TCA GCA AGT GAT  864
Met Asn Thr Ile Gln Gln Leu Met Met Ile Leu Asn Ser Ala Ser Asp  282

CAA CCT TCA GAA AAT CTG ATT TCC TAT TTT AAC AAC TGC ACA GTG AAT  912
Gln Pro Ser Glu Asn Leu Ile Ser Tyr Phe Asn Asn Cys Thr Val Asn  298

CCA AAA GAA AGT ATA CTG AAA AGA GTG AAG GAT ATA GGA TAC ATC TTT  960
Pro Lys Glu Ser Ile Leu Lys Arg Val Lys Asp Ile Gly Tyr Ile Phe  314
```

FIG. 2B

```
AAA GAG AAA TTT GCT AAA GCT GTG GGA CAG GGT TGT GTC GAA ATT GGA 1008
Lys Glu Lys Phe Ala Lys Ala Val Gly Gln Gly Cys Val Glu Ile Gly  330

TCA CAG CGA TAC AAA CTT GGA GTT CGC TTG TAT TAC CGA GTA ATG GAA 1056
Ser Gln Arg Tyr Lys Leu Gly Val Arg Leu Tyr Tyr Arg Val Met Glu  346

TCC ATG CTT AAA TCA GAA GAA GAA CGA TTA TCC ATT CAA AAT TTT AGC 1104
Ser Met Leu Lys Ser Glu Glu Glu Arg Leu Ser Ile Gln Asn Phe Ser  362

AAA CTT CTG AAT GAC AAC ATT TTT CAT ATG TCT TTA TTG GCG TGC GCT 1152
Lys Leu Leu Asn Asp Asn Ile Phe His Met Ser Leu Leu Ala Cys Ala  378

CTT GAG GTT GTA ATG GCC ACA TAT AGC AGA AGT ACA TCT CAG AAT CTT 1200
Leu Glu Val Val Met Ala Thr Tyr Ser Arg Ser Thr Ser Gln Asn Leu  394

GAT TCT GGA ACA GAT TTG TCT TTC CCA TGG ATT CTG AAT GTG CTT AAT 1248
Asp Ser Gly Thr Asp Leu Ser Phe Pro Trp Ile Leu Asn Val Leu Asn  410

TTA AAA GCC TTT GAT TTT TAC AAA GTG ATC GAA AGT TTT ATC AAA GCA 1296
Leu Lys Ala Phe Asp Phe Tyr Lys Val Ile Glu Ser Phe Ile Lys Ala  426

GAA GGC AAC TTG ACA AGA GAA ATG ATA AAA CAT TTA GAA CGA TGT GAA 1344
Glu Gly Asn Leu Thr Arg Glu Met Ile Lys His Leu Glu Arg Cys Glu  442

CAT CGA ATC ATG GAA TCC CTT GCA TGG CTC TCA GAT TCA CCT TTA TTT 1392
His Arg Ile Met Glu Ser Leu Ala Trp Leu Ser Asp Ser Pro Leu Phe  458

GAT CTT ATT AAA CAA TCA AAG GAC CGA GAA GGA CCA ACT GAT CAC CTT 1440
Asp Leu Ile Lys Gln Ser Lys Asp Arg Glu Gly Pro Thr Asp His Leu  474
```

FIG. 2C

```
GAA TCT GCT TGT CCT CTT AAT CTT CCT CTC CAG AAT AAT CAC ACT GCA 1488
Glu Ser Ala Cys Pro Leu Asn Leu Pro Leu Gln Asn Asn His Thr Ala  490

GCA GAT ATG TAT CTT TCT CCT GTA AGA TCT CCA AAG AAA AAA GGT TCA 1536
Ala Asp Met Tyr Leu Ser Pro Val Arg Ser Pro Lys Lys Lys Gly Ser  506

ACT ACG CGT GTA AAT TCT ACT GCA AAT GCA GAG ACA CAA GCA ACC TCA 1584
Thr Thr Arg Val Asn Ser Thr Ala Asn Ala Glu Thr Gln Ala Thr Ser  522

GCC TTC CAG ACC CAG AAG CCA TTG AAA TCT ACC TCT CTT TCA CTG TTT 1632
Ala Phe Gln Thr Gln Lys Pro Leu Lys Ser Thr Ser Leu Ser Leu Phe  538

TAT AAA AAA GTG TAT CGG CTA GCC TAT CTC CGG CTA AAT ACA CTT TGT 1680
Tyr Lys Lys Val Tyr Arg Leu Ala Tyr Leu Arg Leu Asn Thr Leu Cys  554

GAA CGC CTT CTG TCT GAG CAC CCA GAA TTA GAA CAT ATC ATC TGG ACC 1728
Glu Arg Leu Leu Ser Glu His Pro Glu Leu Glu His Ile Ile Trp Thr  570

CTT TTC CAG CAC ACC CTG CAG AAT GAG TAT GAA CTC ATG AGA GAC AGG 1776
Leu Phe Gln His Thr Leu Gln Asn Glu Tyr Glu Leu Met Arg Asp Arg  586

CAT TTG GAC CAA ATT ATG ATG TGT TCC ATG TAT GGC ATA TGC AAA GTG 1824
His Leu Asp Gln Ile Met Met Cys Ser Met Tyr Gly Ile Cys Lys Val  602

AAG AAT ATA GAC CTT AAA TTC AAA ATC ATT GTA ACA GCA TAC AAG GAT 1872
Lys Asn Ile Asp Leu Lys Phe Lys Ile Ile Val Thr Ala Tyr Lys Asp  618

CTT CCT CAT GCT GTT CAG GAG ACA TTC AAA CGT GTT TTG ATC AAA GAA 1920
Leu Pro His Ala Val Gln Glu Thr Phe Lys Arg Val Leu Ile Lys Glu  634
```

FIG. 2D

```
GAG GAG TAT GAT TCT ATT ATA GTA TTC TAT AAC TCG GTC TTC ATG CAG 1968
Glu Glu Tyr Asp Ser Ile Ile Val Phe Tyr Asn Ser Val Phe Met Gln  650

AGA CTG AAA ACA AAT ATT TTG CAG TAT GCT TCC ACC AGG CCC CCT ACC 2016
Arg Leu Lys Thr Asn Ile Leu Gln Tyr Ala Ser Thr Arg Pro Pro Thr  666

TTG TCA CCA ATA CCT CAC ATT CCT CGA AGC CCT TAC AAG TTT CCT AGT 2064
Leu Ser Pro Ile Pro His Ile Pro Arg Ser Pro Tyr Lys Phe Pro Ser  682

TCA CCC TTA CGG ATT CCT GGA GGG AAC ATC TAT ATT TCA CCC CTG AAG 2112
Ser Pro Leu Arg Ile Pro Gly Gly Asn Ile Tyr Ile Ser Pro Leu Lys  698

AGT CCA TAT AAA ATT TCA GAA GGT CTG CCA ACA CCA ACA AAA ATG ACT 2160
Ser Pro Tyr Lys Ile Ser Glu Gly Leu Pro Thr Pro Thr Lys Met Thr  714

CCA AGA TCA AGA ATC TTA GTA TCA ATT GGT GAA TCA TTC GGG ACT TCT 2208
Pro Arg Ser Arg Ile Leu Val Ser Ile Gly Glu Ser Phe Gly Thr Ser  730

GAG AAG TTC CAG AAA ATA AAT CAG ATG GTA TGT AAC AGC GAC CGT GTG 2256
Glu Lys Phe Gln Lys Ile Asn Gln Met Val Cys Asn Ser Asp Arg Val  746

CTC AAA AGA AGT GCT GAA GGA AGC AAC CCT CCT AAA CCA CTG AAA AAA 2304
Leu Lys Arg Ser Ala Glu Gly Ser Asn Pro Pro Lys Pro Leu Lys Lys  762

CTA CGC TTT GAT ATT GAA GGA TCA GAT GAA GCA GAT GGA AGT AAA CAT 2352
Leu Arg Phe Asp Ile Glu Gly Ser Asp Glu Ala Asp Gly Ser Lys His  778

CTC CCA GGA GAG TCC AAA TTT CAG CAG AAA CTG GCA GAA ATG ACT TCT 2400
Leu Pro Gly Glu Ser Lys Phe Gln Gln Lys Leu Ala Glu Met Thr Ser  794
```

FIG. 2E

```
ACT CGA ACA CGA ATG CAA AAG CAG AAA ATG AAT GAT AGC ATG GAT ACC   2448
Thr Arg Thr Arg Met Gln Lys Gln Lys Met Asn Asp Ser Met Asp Thr    810

TCA AAC AAG GAA GAG AAA TGA GGATCTCAGG ACCTTGGTGG ACACTGTGTA      2499
Ser Asn Lys Glu Glu Lys ***

CACCTCTGGA TTCATTGTCT CTCACAGATG TGACTGTATA ACTTTCCCAG GTTCTGTTTA 2559

TGGCCACATT TAATATCTTC AGCTCTTTTT GTGGATATAA AATGTGCAGA TGCAATTGTT 2619

TGGGTGATTC CTAAGCCACT TGAAATGTTA GTCATTGTTA TTTATACAAG ATTGAAAATC 2679

TTGTGTAAAT CCTGCCATTT AAAAAGTTGT AGCAGATTGT TTCCTCTTCC AAAGTAAAAT 2739

TGCTGTGCTT TATGGATAGT AAGAATGGCC CTAGAGTGGG AGTCCTGATA ACCCAGGCCT 2799

GTCTGACTAC TTTGCCTTCT TTTGTAGCAT ATAGGTGATG TTTGCTCTTG TTTTTATTAA 2859

TTTATATGTA TATTTTTTTA ATTTAACATG AACACCCTTA GAAAATGTGT CCTATCTATC 2919

ATCCAAATGC AATTTGATTG ACTGCCCATT CACCAAAATT ATCCTGAACT CTTCTGCAAA 2979

AATGGATATT ATTAGAAATT AGAAAAAAAT TACTAATTTT ACACATTAGA TTTTATTTTA 3039

CTATTGGAAT CTGATATACT GTGTGCTTGT TTTATAAAAT TTTGCTTTTA ATTAAATAAA 3099

AGCTGGAAGC AAAGTATAAC CATATGATAC TATCATACTA CTGAAACAGA TTTCATACCT 3159

CAGAATGTAA AAGAACTTAC TGATTATTTT CTTCATCCAA CTTATGTTTT TAAATGAGGA 3219

TTATTGATAG TGG                                                    3232
```

FIG. 2F

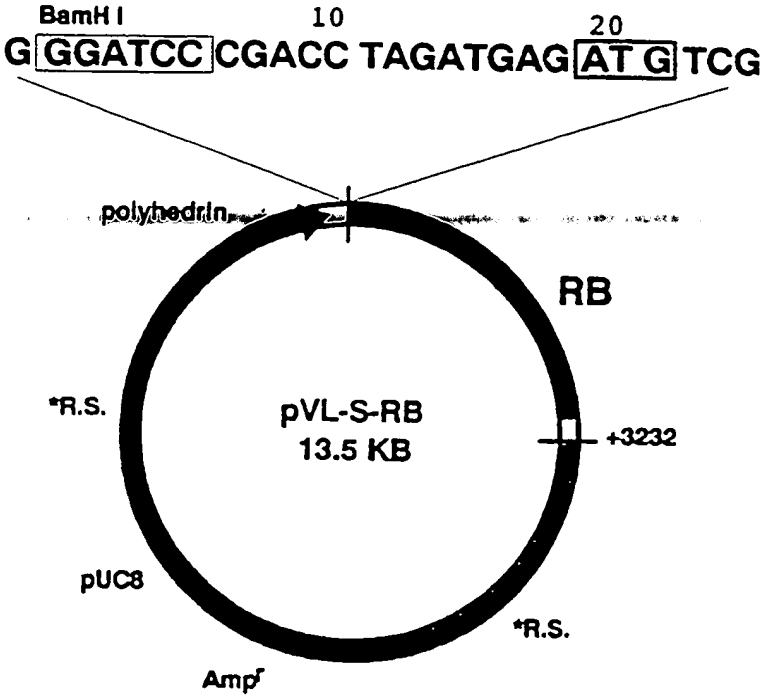

BamH I
10
20
G GGATCC CGACC TAGATGAG ATG TCG
polyhedrin
RB
*R.S.
pVL-S-RB
13.5 KB
+3232
pUC8
*R.S.
Amp^r

Figure 3.

FIG. 4C

FIG. 4B

FIG. 4A

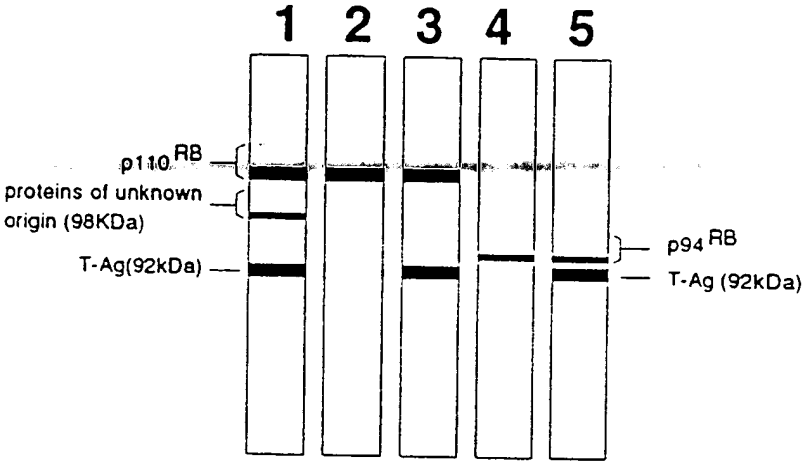
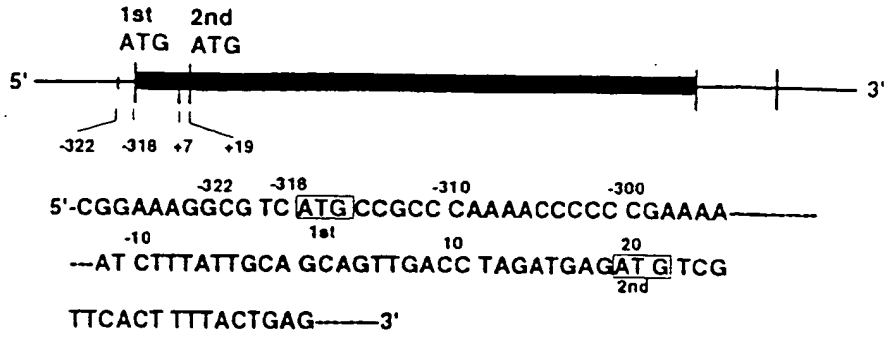

1
2
3
4
5
p110 RB
proteins of unknown origin (98KDa)
T-Ag(92kDa)
p94 RB
T-Ag (92kDa)

Figure 5.

A. RB cDNA



B. RB Expression Plasmids

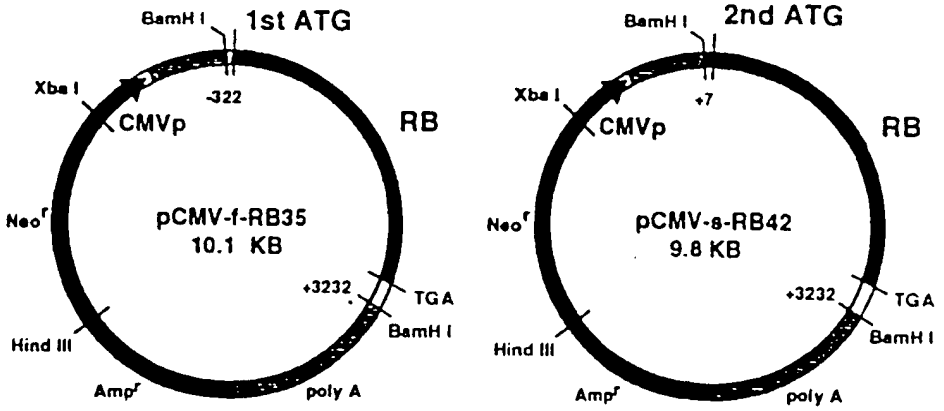


Figure 6.

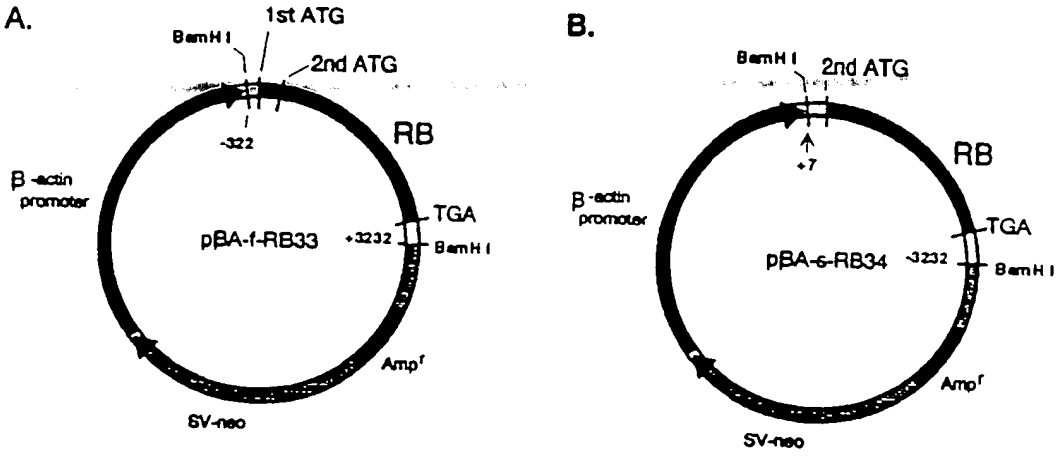

A.
BamH I
1st ATG
2nd ATG
-322
RB
β -actin promoter
pβA-f-RB33
+3232
TGA
BamH I
Amp^r
SV-neo
B.
BamH I
2nd ATG
+7
RB
β -actin promoter
pβA-6-RB34
-3232
TGA
BamH I
Amp^r
SV-neo

Figure 7.

FIG. 8C

FIG. 8B

FIG. 8A

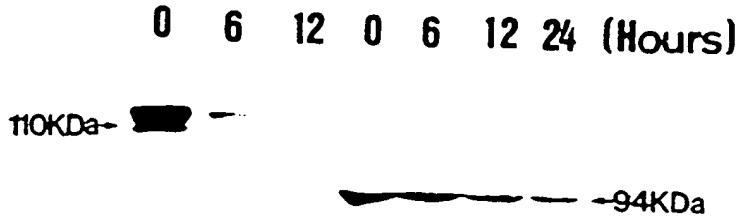


FIG. 9

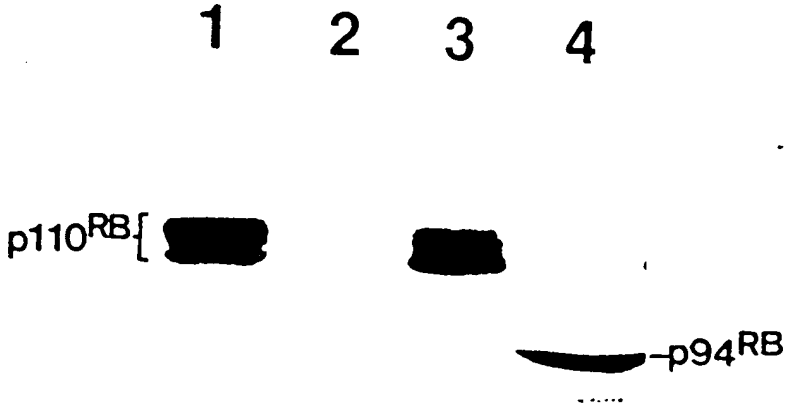


FIG. 10

FIG. 11A FIG. 11B FIG. 11C

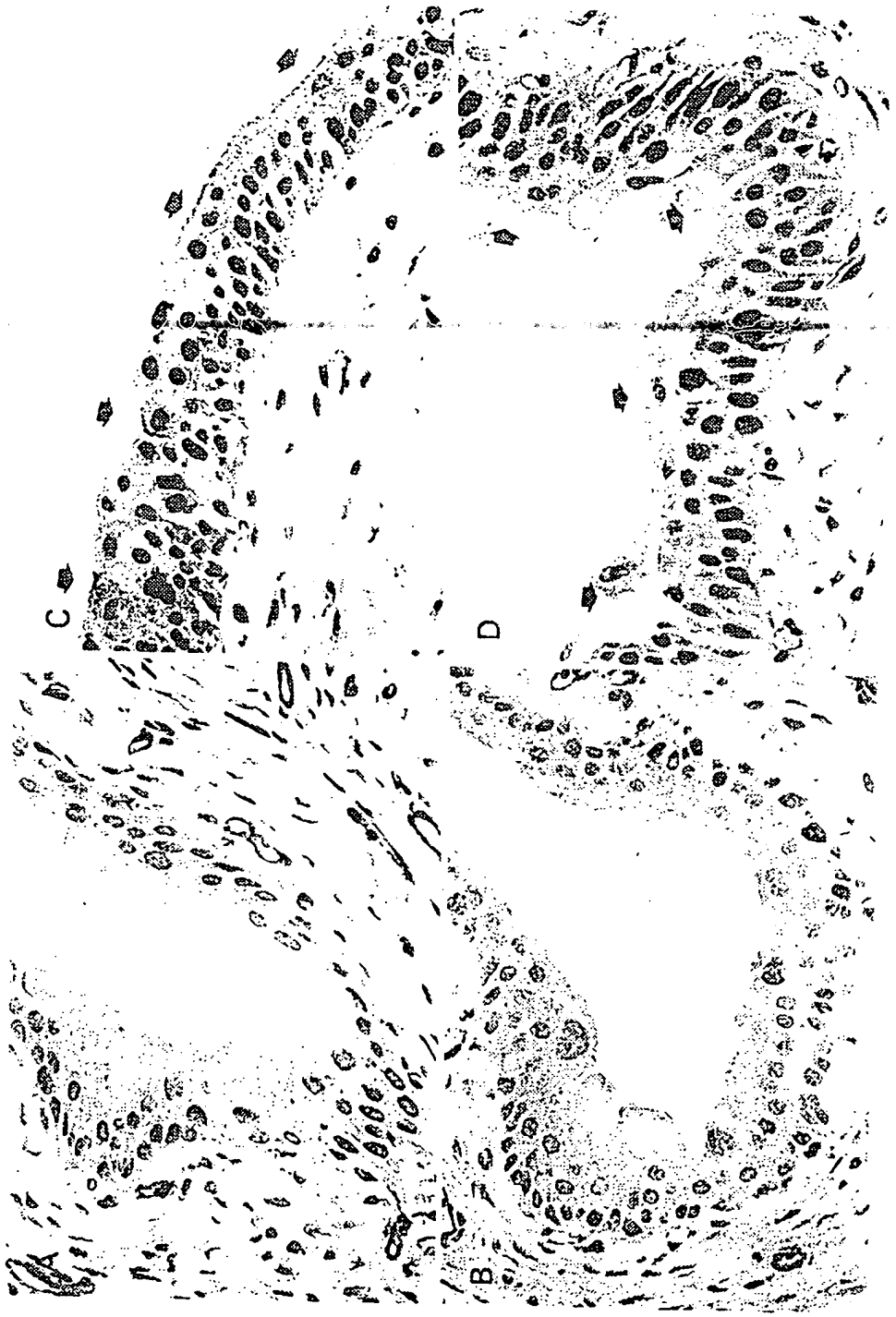


FIG.12A

FIG.12B

FIG.12C

FIG.12D

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :435/69.1, 172.3; 514/2, 44; 530/350; 536/24.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.3; 514/2, 44; 530/350; 536/24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, Volume 249, issued 14 September 1990, E.G. Nabel et al., "Site-specific gene expression in vivo by direct gene transfer into the arterial wall", pages 1285-1288, see entire article.	1-30, 42 and 53
Y	SCIENCE, Volume 235, issued 13 March 1987, W.-H. Lee et al., "Human retinoblastoma susceptibility gene: cloning, identification, and sequence", pages 1394-1399, see entire article.	1-37 AND 40-53
Y	SCIENCE, Volume 254, issued 29 November 1991, R.J. Clem et al., "Prevention of apoptosis by a baculovirus gene during infection of insect cells", pages 1388-1390, see entire article.	36, 37, 40 and 41

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 07 JUNE 1994	Date of mailing of the international search report JUL 11 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRIAN R. STANTON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NATURE, Volume 360, issued 12 November 1992, D.W. Goodrich et al., "Abrogation by c-*myc* of G1 phase arrest induces by *RB* protein but not by p53", pages 177-179, see entire document.	1-35 and 42-53
Y	NATURE, Volume 329, issued 15 October 1987, W.-H. Lee et al., "The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity", pages 642-645, see entire article.	1-37 and 40-53
Y	ONCOGENE, Volume 6, Number 7, issued 1991, H.-J. Xu et al., "Lack of nuclear RB protein staining in G0/middle G1 cells: correlation to changes in total RB protein level", pages 1139-1146, see entire article.	1-37 and 40-53
Y	CANCER RESEARCH, Volume 52, Number 22, issued 15 November 1992, A. Banerjee et al., "Changes in growth and tumorigenicity following reconstitution of retinoblastoma gene function in various human cancer cell types by microcell transfer of chromosome 13", pages 6297-6304, see entire article.	1-37 and 40-53
Y	JOURNAL OF CLINICAL INVESTIGATION, Volume 85, Number 4, issued April 1990, W.F. Benedict et al., "Role of the retinoblastoma gene in the initiation and progression of human cancer", pages 988-993, see entire article.	1-37 and 40-53
Y	THE JOURNAL OF EXPERIMENTAL MEDICINE, Volume 169, number 1, issued 01 January 1989, H. Karasuyama et al., "Autocrine growth and tumorigenicity of interleukin 2-dependent helper T cells transfected with IL-2 gene", pages 13-25, see entire article.	1-37 and 40-53
Y	CELL, Volume 68, issued 10 January 1992, M.A. Rosenfeld et al., "In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium", pages 143-155, see entire article.	1-30, 42 and 53
Y	HUMAN GENE THERAPY, Volume 2, issued 1991, K.W. Culver et al., "Lymphocyte gene therapy", pages 107-109, see entire article.	1-30, 42 and 53
Y	ONCOGENE, Volume 4, Number 4, issued April 1989, A. T'Ang et al., "Genomic organization of the human retinoblastoma gene", pages 401-407, see entire article.	1-37 and 40-53

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ONCOGENE, Volume 4, issued 1989, H.-J. Xu et al., "The retinoblastoma susceptibility gene product: a characteristic pattern in normal cells and abnormal expression in malignant cells", pages 807-812, see entire article.	1-30 and 42-53
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Volume 85, Number 16 issued August 1988, E. Y.-H. P. Lee et al., "Molecular mechanism of retinoblastoma gene inactivation in retinoblastoma cell line Y79", pages 6017-6021, see entire article.	1-35 and 42-53
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Volume 88, Number 12 issued 15 June 1991, R. Takahashi et al., "The retinoblastoma gene functions as a growth and tumor suppressor in human bladder carcinoma cells", pages 5257-5261, see entire article.	1-35 and 42-53
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Volume 84, Number 24, issued December 1987, S.H. Friend et al., "Deletions of a DNA sequence in retinoblastomas and mesenchymal tumors: Organization of the sequence and its encoded protein", pages 9059-9063, see entire article.	1-37 and 40-53
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, Volume 84, Number 14 issued July 1987, P. Gunning et al., "A human beta-actin expression vector system directs high-level accumulation of antisense transcripts", pages 4831-4835, see entire article.	1-30, 42 and 53
Y	WO, A, 92/22640 (TAKAHASHI ET AL.) 23 DECEMBER 1992, see entire patent application.	1-37 and 40-53
Y	EP, A, 0,293,266 (FUNG ET AL.) 30 NOVEMBER 1988, see entire patent application.	1-37 and 40-53
Y	WO, A, 90/05180 (LEE ET AL.) 17 MAY 1990, see entire patent application.	1-37 and 40-53
Y	WO, A, 91/15580 (FUNG ET AL.) 17 OCTOBER 1991, see entire patent application.	1-37 and 40-53
Y	NATURE GENETICS, Volume 1, issued August 1992, J.H. Wolfe et al., "Herpesvirus vector gene transfer and expression of beta-glucuronidase in the central nervous system of MPS VII mice", pages 379-384, see entire article.	1-37 and 40-53

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 38 and 39
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 38 and 39 have not been searched because the preambles of said claims recite that methods are claimed and said claims depend from a product claim (claim 32). Therefore it is not possible to determine what process is intended.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A01N 1/00; A61K 31/70, 37/00; C07H 1/00, 3/00, 13/00; C12N 5/00, 15/00; C12P 21/06

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Databases: APS; Biosis Previews; DissAbs; LifeSci; Pascal; HealthPeriodical DB; Medline; Toxline; Cancerlit; Derwent World Patents; Derwent Biotechnology Abs; SciSearch; Genbank

Search terms: p94; retinoblast?; canc?; tumor?; gene?; neoplas?; p110; rb; Xu?/au; Benedict?/au

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I. Claims 1-30 and 53, drawn to DNA encoding the retinoblastoma (Rb) gene and methods of using said DNA for in vivo gene therapy.

Group II. Claims 31-35 , drawn to Rb proteins.

Group III. Claims 36, 37, 40 and 41, drawn to a method of making Rb proteins.

Group IV. Claim 42, drawn to ex vivo gene therapy using genes encoding the Rb gene product.

Group V. Claims 43-51, drawn to in vivo therapy using Rb proteins.

Group VI. Claim 52, drawn to ex vivo therapy using Rb proteins.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Any of Groups I or IV-VI and Group III do not relate to a single inventive concept because they are drawn to materially different methods that require divergent areas of search and consideration. For example, the methods of Groups I and IV-VI relate to therapeutic applications which require consideration of appropriate administration routes, selection of suitable adjuvants, methods of targeting therapeutics to appropriate tissue and means of determining efficacy of treatment. Such considerations are not required for methods of making proteins (Group III).

Either of Groups I or V and either of Groups IV or VI do not relate to a single inventive concept because they are drawn to materially different methods that require divergent areas of search and consideration. For example, ex vivo therapy (Groups IV and VI) requires consideration of means of identifying and isolating appropriate target cells, analysis of culture means useful for maintaining said cells ex vivo and consideration of means of administering cellular (Groups IV and VI) versus isolated nucleic acids or proteins (Groups I and V, respectively) compositions.

Either of Groups I or IV and either of Groups V or VI do not relate to a single inventive concept because they are drawn to materially different methods that require divergent areas of search and consideration. For example, the former two methods utilize nucleic acids as therapeutic agents which require consideration of in vivo or intracellular gene expression. Such considerations include selection of suitable promoters and transcriptional and translational signal elements, as well as analysis of the tissue and cell type specificity of said promoter. Such considerations are not required for analysis of protein therapeutic agents (Groups V and VI).

Any of the therapeutic methods of Groups I or IV-VI and the proteins of Group II do not relate to a single inventive concept because the former groups relates to therapeutic applications which require considerations listed above and none of said considerations and corresponding areas of search are required for the analysis of proteins per se as claimed within the invention of Group II.

The proteins of Group II and the methods of making proteins of Group III do not relate to a single inventive concept because proteins may be made by a materially different process from that using recombinant DNA technology

(Group III), such as by direct chemical synthesis and because the methods of Group III require consideration of appropriate gene expression systems and such consideration is not required for analysis of proteins (Group II) per se.